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IMPROVED BACTERIAL NANOCELLULOSE PRODUCTION BY CO-CULTIVATION

Master of Science Thesis

Examiner: Assistant prof. Ville Santala and Dr. Rahul Mangayil
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ABSTRACT

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Bacterial cellulose has high degree of crystallinity, high purity, excellent water-binding properties and good shape retention advantages, compared to plant cellulose, and it has many potential applications in various industries. The gluconic acid, a by-product produced during BC production, can decrease the pH of cultivation that leads to low BC yield by *Komagataeibacter xylinus*. Thus, maintaining the pH of cultivation for BC production was important and urgent task. In this study, the *Acinetobacter baylyi ADP1Δgcd* was employed to co-cultivate with *K. xylinus*, which aimed to well control the pH suitable for BC production by consuming the gluconic acid accumulation during static cultivation.

The *K. xylinus* engineered type (pABCD) and wild type (WT) was proved to be able to produce BC in MA/9 medium. And the pABCD produced 2-fold more BC production than that of WT in MA/9 medium after 5 days. pABCD and WT obtained lower BC productivity in MA/9 medium than that in HS medium. The effect of different concentration glucose addition for growth of pABCD and BC production was also investigated in this study. The highest BC production on 2% glucose addition was around 0.267 g/L produced by pABCD after 5 days cultivation in MA/9 medium, and followed by 5% glucose (0.266 g/L), 1% glucose (0.2 g/L), 3.5% glucose (0.167 g/L) and 0.5% glucose (0.133 g/L). So, the BC productivity of pABCD increased with the increasing of glucose concentration until 2% in MA/9 medium.

Moreover, the effect of arabinose (1%) and initial inoculate volume for BC production was studied. The presence of arabinose (1%) was proved to be able to achieve an increase of BC biosynthesis in MA/9 medium by inducing overexpression of bcs operon in pABCD strains. It yielded 0.267 g/L BC, which was 2-fold higher than that of arabinose absence. And the initial inoculate volume (OD_{600}=0.02 or 0.04) of pABCD has no significant effect on the final BC productivity in MA/9 medium. The maximum concentration of gluconic acid consumed by *A. baylyi ADP1Δgcd* was about 80 mM in MA/9 medium. It was demonstrated that *A. baylyi ADP1Δgcd* was able to utilize all gluconic acid accumulated in MA/9 medium supplemented with glucose (2%) after 5 days cultivation. Finally, the pH of co-cultivation was maintained at optimized range (5.0-7.0), compared to that of pABCD pure cultivation group (below 4.0). However, the co-cultivation with and without 50 μg/ml chloramphenicol addition, yield approximately 0.267 g/L and 0.1667 g/L BC after 5 days cultivation, respectively, which was much less than that of pABCD pure cultivation group. The *A. baylyi ADP1Δgcd* helped in eliminating the gluconic acid and maintaining pH, but the glucose consumption was lower in co-cultivation than in pABCD pure cultivation. This phenomenon is interesting and will be a subject of future study.

PREFACE

This thesis, based on the experiment part, which is conducted at the Department of Chemistry and Bioengineering, Tampere University of Technology, Finland, from 1st November 2016 to 1st April, 2017. I am sincerely grateful to my supervisor, Assistant prof. Ville Santala and Dr. Rahul Mangyil. They give me an opportunity to be one of the members of their research group and conduct my thesis work during this period. From the determination of the topic to arrangement of experiments, Assistant prof. Ville Santala has given me great help and useful guidance. Specially, I also would like to thank Dr. Rahul Mangyil, he gave me much useful advices when I encounter difficulties during experiment period, and he also discussed with me about the results of experiment timely.

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May, 2017, Tampere

Tingting Zhan

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LIST OF SYMBOLS AND ABBREVIATIONS

A. baylyi ADP1	*Acinetobacter baylyi ADP1*
A. baylyi ADP1 Δgcd	*Acinetobacter baylyi ADP1 Δgcd*
ATP	Adenosine 5’ triphosphate
BC	Bacterial cellulose
bcs operon	Bacterial cellulose synthase operon
$CaCl_2$	Calcium chloride
CAM	Chloramphenicol
c-di-GMP	Bis-cyclic diguanylate
Fe	Iron
$FeCl_2$	Iron (II) chloride
Glc-6-P	Glucose-6-phosphate
GDH	Membrane bound glucose dehydrogenase gene
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
HS medium	Hestrin and Schramn medium
K. xylinus	*Komagataeibacter xylinus*
KH_2PO_4	Potassium hydrogen phosphate
LA plate	LB medium with agar
LB medium	Lysogeny broth medium
MA/9 medium	Minimal salt medium
$MgSO_4$	Magnesium sulfate
MQ	Milli-Q water
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
OD_{600}	Optical density at 600 nm
UDPGlc	UDP-glucose
UDP-glucose	Uridyl-diphosphate glucose
WT	*K. xylinus* Wild type

1. INTRODUCTION

The cellulose, the most abundant particular biopolymers, is commonly produced by plant sources. Generally speaking, each cellulose has about 9,000 to 10,000 unit polymerization and can afford almost 16.9 GPa and 2 GPa for its tensile and elastic modulus, respectively (Kumar et al.2009). Thus, cellulose can be used as reinforcement material due to its excellent mechanical characteristic. The plant based cellulose is in formed of lignocellulosic biomass which contains lignin, pectin, hemicellulose accompanying with the structure of cell wall (Jonas and Farah 1998). Each year, there are enormous amount of cellulose being needed (approximately 10^{16} kg) and pretreated harshly to remove the lignin composition to obtain the relatively purity cellulose. During this pretreatment process, it can consume huge amount of energy and also bring addition toxic sulfoxides products (Jozala AF et al. 2016; Lee KY et al. 2014).

Compared to cellulose produced by plant, BC has many advantages. For instance, BC extracellularly produced by bacterium is more pure than that of cellulose from plant (Gallegos et al. 2016), so, it is not necessary to perform harshly pretreatment process. And BC has high water absorption, good adhere and moisture ability owe to its thin nanofiber (20-100 nm diagram) and large surface area per unit properties (Fu et al. 2013). And it also has excellent typical properties, like good elastic, tensile strength, insolubility, biodegradability, biocompatibility, durability and nontoxic (Thompson & Hamilton 2001). Those advantages make the BC ideal for production with high economic value. Therefore, BC is considered to be the best biopolymer cellulose, and cellulose biosynthesis of bacterial is also considered to be an effective and convenient method for producing pure and biodegradable cellulose, compared to cellulose from plant (Jozala AF et al. 2106). It has been reported that the extracellular BC production is mostly produced by *Komagataeibacter, Enterobacter, Rhodococcus* and *Sarcina* bacterial genera (Hungund BS et al. 2010; Tanskul S et al. 2013; Schramm M et al.1954). The reasonable inference of BC production from bacteria is that this extracellular polymer can provides protection from external physicochemical stresses and the possibility to sustain in aerobic environment (Schramm M et al.1954; Scott Williams WS et al. 1989). Among bacteria, Komagataeibacter genus is considered the most efficient cellulose producers, and the *K. xylinus* strains is considered to be representative model organism used for investigating BC production ((Masaoka S et al. 1993).

Ross P et al. (1991) reported that BC is synthesized by BC synthase (bcs) operon consisting of four genes – bcsA, bcsB, bcsC and bcsD. The binding of cyclic di-guanosine monophosphate (c-di-GMP) to the cellulose synthase regulatory subunit (bcsB) commences the

cellulose biogenesis. This activates the catalytic subunit (bcsA) to synthesize and polymerize linear β-(1,4) glucan chains from uridine diphosphate glucose (UDP-glucose). Subsequently, the cellulose fibrils are crystallized and secreted to extracellular matrix by bcsD and bcsC, respectively.

The reason of BC attracting many researchers attention is that it can be applied in various industries, such as food, paper, cosmetics, medicine, energy industries and so on (Jozala et al. 2016). Even though, the widely economic application of BC is greatly restricted due to low BC productivity with high cost (Mangyil et al. in press). To surpass this challenge, most of researchers focus on how to improve the BC production for obtaining high BC yield, once it achieves the goal of high-yield BC product with low cost, it would address the dilemma of demand exceeds supply. In previous reviewed articles, there are many factors influencing the productivity and morphology of BC, such as bacterium strains (BC producer), pH of culture medium, temperature of cultivation, culture medium, oxygen content, bioreactor type and culture time. Kuo et al (2015) reported that the glucose is most often used as carbon sources during BC biosynthesis process, even though, BC yield might be low due to the presence of glucose dehydrogenase, which converts glucose into gluconic acid, hence making the pH of culture medium decrease rapidly and potential influencing the BC biosynthesis (Kuo et al 2015). Therefore, eliminating the gluconic acid inhibition effect to maintain pH of culture medium during BC biosynthesis process, is becoming urgently and important task. *A. baylyi* ADP1Δgcd is the laboratory strains, and its gcd gene was removed for glucose dehydrogenase. This gene is present for catalyzing the glucose catabolism at first step. Kannisto M et al. (2015) reported that the engineered *A. baylyi* ADP1Δgcd strains was unable to utilize main sugar of lignocellulosic hydrolysates such as glucose and arabinose, but it has capability to consume gluconate sodium quickly (Kannisto M et al. 2015).

In this study, *A. baylyi* ADP1Δgcd was investigated to co-cultivate with *K. xylinus* for improving BC production, attempted to maintain pH of cultivation by consuming gluconic acid accumulated in MA/9 culture medium, and this strategy also has not been studied yet. To the authors' knowledge, the defined medium (MA/9 minimum medium), a low cost and a simple component culture medium, has not been studied yet for BC production from *K. xylinus* WT strains and pABCD strains. Before doing this co-cultivation experiment, there are several pre-test experiments should be conducted at first, to support co-cultivation experiment. Firstly, the BC producing capability of wild type and engineered types of *K. xylinus* strain was investigated in HS medium and MA/9 medium, optimization strain with high BC yield ability chose could be employed in next experiment. Secondly, the effect of glucose concentration and arabinose for BC production and cells growth also should investigate in this study. Meanwhile, the amount of gluconic acid accumulated during BC production process should be calculated by HPLC analysis method. Thirdly, *A. baylyi ADP1Δgcd* consumed gluconic acid ability also should be studied and known. Last but not the least, the *K. xylinus* co-cultivates with *A. baylyi*

ADP1Δgcd, and then its cell growth and BC yield are investigated, including analysis of glucose utilization for BC production and gluconic acid consumed by the *A. baylyi ADP1Δgcd*. Further, the methodology of *K. xylinus* co-cultivated with *A. baylyi ADP1Δgcd* has never been practiced and discussed yet.

2. THEORETICAL BACKGROUND

2.1 Cellulose (plant based cellulose)

Cellulose is the most abundant renewable polymer in the world (Dufresne A 2007), and it is synthesized by a number of higher to lower plants. The structural material of cellulose is organized as microfibrils linked together to form cellulose fibers (Siqueira G et al. 2010). Cellulose is made of a linear homopolysaccharide composed of β-D-glucopyranose units linked together by β-1-4-linkages (Brännvall E 2007). Each monomer stands three hydroxyl groups. So, these hydroxyl groups play a significant role in directing the crystalline packing and also governing the physical properties of cellulose (John MJ and Thomas S 2008). The plant based cellulose is in formed of lignocellulosic biomass containing lignin, pectin, hemicellulose accompanying with the structure of cell wall (Jonas and Farah 1998). Every year, there are approximately 10^{16} kg cellulose being needed and pretreated harshly to remove the lignin composition to obtain the relatively purity cellulose. During this pretreatment process, it can consume huge amount of energy and also bring addition toxic sulfoxides products (Jozala AF et al. 2016; Lee KY et al. 2014). In Figure 1, it shows the cellulose cell wall scheme and the microfibril organisms.

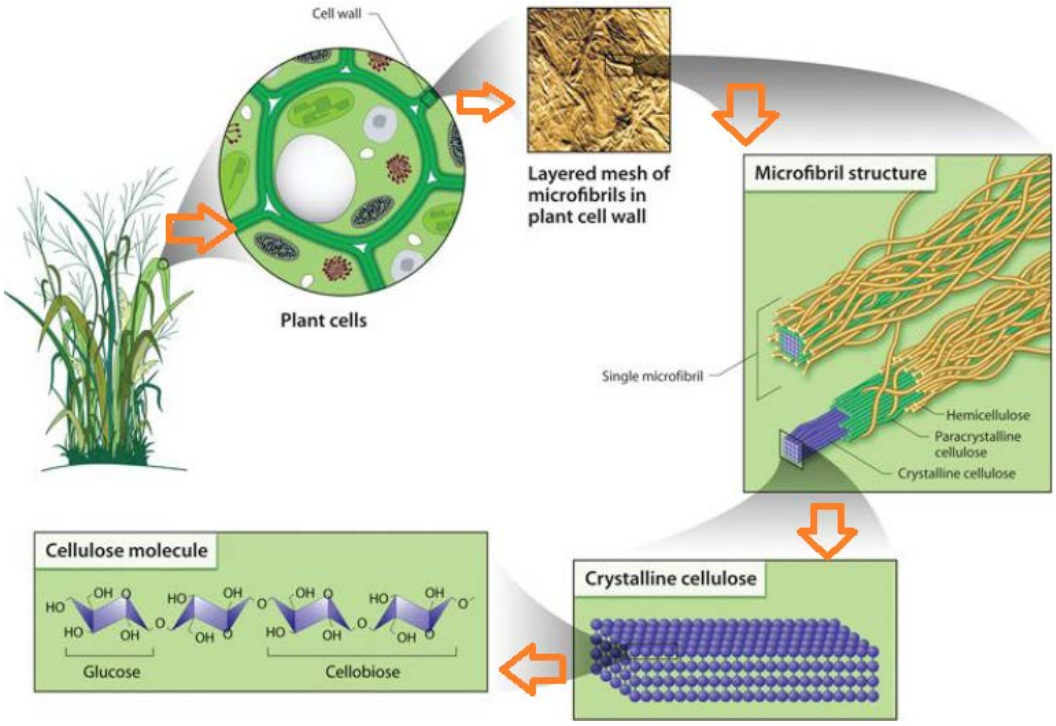
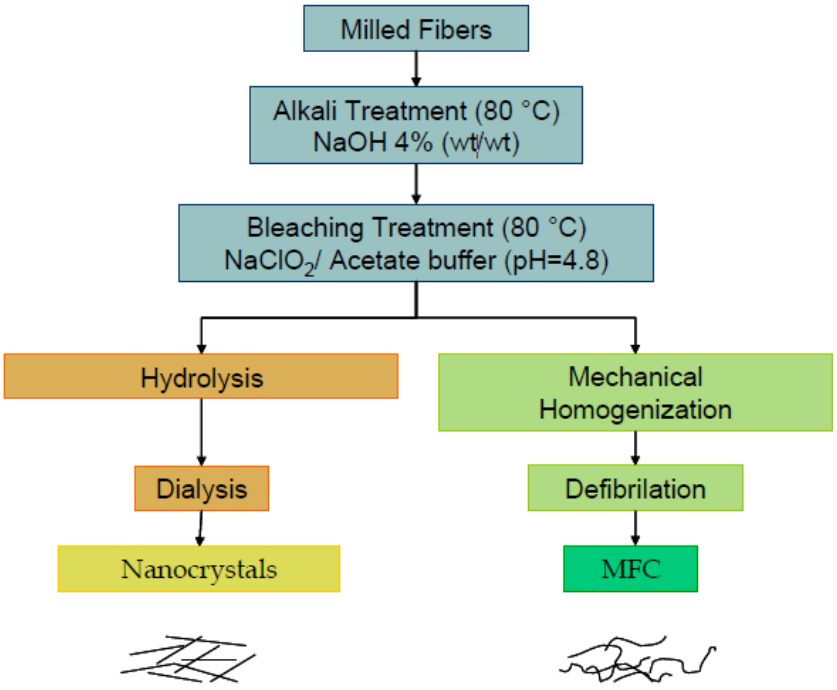


Figure 1.*Cellulose cell wall scheme and microfibril organization (adapted from the Siqueira G et al. 2010)*

In order to obtain the cellulose nanocrystals from plant organisms, cellulosic biomass must be pretreated through several steps (shown in Figure 2).



B

Figure 2. *Main steps involved in the preparation of cellulose nanoparticles (adapted from Siqueira G et al. 2010).*

Besides that, there are various pretreatment methods used for obtaining cellulose nancrystals. Pretreatment technologies for cellulosic biomass include biological, mechanical, chemical methods and combined chemical and mechanical methods (Harmsen PFH et al. 2010). But, each of them has more or less drawbacks. Biological pretreatments employ microorganisms which degrade the lignin and hemicellulose, and the main disadvantage of biological treatments is that the hydrolysis rate is quite low and the cellulose also can be attacked by microorganism (Harmsen PFH et al. 2010). Mechanical methods like grinding and milling often consume high energy and capital costs (Harmsen PFH et al. 2010). Chemical pretreatments that use chemicals like alkalis, ozone, peroxide, aim to remove lignin, which lead to an enhanced cellulose enzymatic degradability (Harmsen PFH et al. 2010). For example, ozo-nolysis requires high amount of O3, which is expensive, so this pretreatment method would be less cost-effective (Harmsen PFH et al. 2010). Therefore, obtaining cellulose nanocrystals from plant biomass is less cost-effective way according to its limitation of cellulose pretreatment methods, hence, 'No- pretreatment' cellulose like BC is emergently demanded recently.

2.2 Bacterial cellulose

2.2.1 Introduction of Bacterial cellulose

BC was discovered by brown in 1886. He found that the mycoderma aceti had capability to produce one extra strong membrane cellulose when it was cultivated in medium with fructose (Brown AJ 1886). Brown AJ (1886) also found that this cellulose looked like a Jell transparent film membrane and attained to about 25 mm thickness. Apart from the plant cellulose, BC is biosynthesized by some certain bacteria, such as *Komagataeibacter* spp., *Agrobacterium* spp., *Rhizobium* spp., and *Alcaligenes* spp. (Vandamme et al. 1998). Son et al. (2010) described that the *K. xylinum* strains could be able to produce BC by utilizing carbon sources at 25℃ to 30℃ and pH at around 4.5 to 7.5. The reasonable hypothesis reported by several authors explained why the BC was produced by some bacterium: 1) to keep connect to the surface of culture medium in high oxygen condition (Gromet et al. 1957); 2) to prevent the x-ray or strong sunlight (Williams et al. 1989); 3) to prevent the heavy metal ions and transport nutrient through diffusion way (Iguchi et al. 2000). Donini (2010) also reported that the BC had capability to be produced higher efficiently by microorganism than that of plant cellulose. For instance, in order to yield 80 t cellulose, the eucalyptus will take 7 years to yield those amount of cellulose contents, in contrast, the microorganism only will take approximately 22 days to achieve this goal in bioreactors (Donini et al. 2010) Moreover, the BC has attracted many scientists' interest due to its excellent properties such as high purity, nanoscale in diameter, excellent mechanical strength, chemical stability, biodegradability, bio compatibility and crystalline network structure and so on (Jozala et al. 2016). Thus, the BC can be applied in various industries, even though, the spread commercial applicability of BC is still restricted due to the low BC yield (Mangyil et al. In press). Therefore, it is necessary and important to develop method to optimize the BC production process. Before doing this study, it is necessary to know the structure and composition of BC, the metabolism pathway of the BC production, as well as the possible factors influencing BC production and fermentation process.

2.2.2 Composition and structure of BC

BC mostly is left-hand twisted produced, and the diameter and length of its individual fiber range from 25-100 nm and 1 mm, respectively (Colvin JR 1980). So, those fibers are also called nanofibers. Unlike cellulose produced by plants, BC is no need to do fibrillated treatment to obtain nanofibrillated cellulose, since BC has well aligned BC nanofibers naturally (Herrick FW et al. 1983). Importantly, BC is excellent standard nanocellulose in the world, because it has good nanoscale nanofibers, and it could be disintegrated into smallest fibers approximately only 6-7 nm in diameter, and those smallest and

perfect fibers are called elementary fibrils (Frey-Wyssling A 1953, cited in Lee KY et al. 2014).

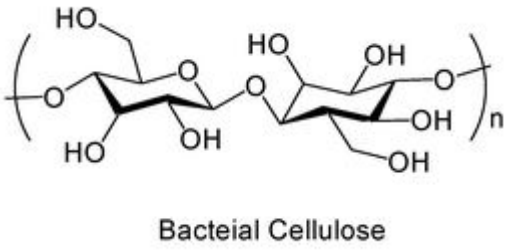


Figure 3. Chemical structure of BC (n= the polymerization degree) (adapted from Shi ZJ et al. 2012)

As can be seen in Figure 3, it shows the molecular formula of BC and there are several D-glucose blocks connected repeatedly, which is made up the complex and crystalized cellulose structure. Jonas and Farah (1998) have reported that the degree of polymerization of BC is around 2,000 to 6,000. In contrast, the polymerization degree of plant cellulose is up to 13,000 to 14,000, which is almost 2- 6 folds more than that of the BC (Jonas and Farah 1998).

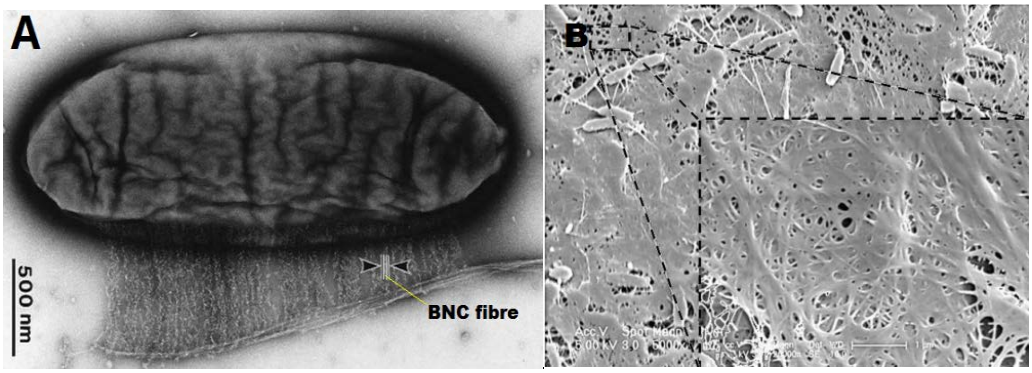


Figure 4. *A) The Scanning electron microscopy (SEM) of a K. xylinus bacteria producing a cellulose fiber (adapted from Hirai et al. 2002); B) An image of a bacteria-generated BC network scanned by electron microscope (Iqbal 2015).*

Further, BC has typical properties, such as degradability, biocompatibility and easy to be modified chemical ability (Czaja et al. 2007) owing to many OH groups inside of BC structure. For the structure of cellulose, there are two regions: ordered and disordered domains (Lanyon YH et al. 2005). The disordered domains mainly form fine fiber at the disordered micro-fibrils surface. While, the ordered domains are mostly transparent (Lanyon YH et al. 2005). In Figure 4 A), a 24 nm strand is represented between the arrowheads, and then the ribbon formed by some of them, is being assembled. The BC networked is observed in Figure 4 B).

As can be seen from Table 1, *A. xylinum* was able to produce extracellular pellicle composed of ribbons by extracellular process. In contrast, the structure of BC produced by *Achromobacter, Aerobacter, Alcaligenes* or *Agrobacterium and Rhibozium* genus bacterium produce are fibrils or short fibrils. Specially, the BC, which was produced by Sarcina genus bacteria, has amorphous cellulose structure (Jonas and Farah 1998).

Table 1. *The different types of BC producers (adapted from the Jonas and Farah 1998).*

Genus	**Cellulose structure**
Komagataeibacter	Extracellular pellicle composed of ribbons
Achromobacter	fibrils
Aerobacter	Short fibrils
Agrobacterium	fibrils
Alcaligenes	fibrils
Sarcina	Amorphous cellulose

2.2.3 Biosynthesis of BC

I. Metabolism pathway of BC biosynthesis

Unlike the cellulose formation in plants, the *K. xylinus* bacterium is a well and efficient extracellular BC producer compared with other genus strains existed in the world (Kongruang S 2008). It is also known as the *Acetobacter xylinum or Gluconoacetobacter xylinus* and studied mostly for few years, because it is the highest BC producer and able to utilize variety carbon sources for cellulose synthesis (Kongruang S 2008). For example, glucose is one of common carbon sources utilized in cellulose biosynthesis, which is not only used as energy sources but also transformed into cellulose (Kuo et al. 2015). Another important reason is that the gene for BC biosynthesis from this bacterium genome has been identified, which helps us to understand cellulose biosynthesis mechanism clearly (Kongruang S 2008). That is why *K. xylinus* is the most common bacteria used for producing BC and also attract other insight into gene organization for improve BC productivity. From the Table 2. it lists various BC producer bacteria, carbon sources and other common supplements, often used for producing BC.

Table 2. *The different cellulose bacteria producers used for producing BC (adapted from Chaw et al. 2009)*

Bacteria	**Carbon source**	**supplement**	**Culture time (h)**	**Yield (g/L)**
A. xylinum BRC 5	Glucose	Ethanol+oxygen	50	15.30
Acetobacter sp. V6	Glucose	Ethanol	192	4.16
Acetobacter sp. A9	Glucose	Ethanol	192	15.20
G. xylinus K3	Mannitol	Green tea	168	3.34
G. xylinus IFO 13773	Glucose	Lignosulfonate	168	10.10
A. xylinum E25	Flucose		168	3.50
A. xylinum BPR2001	Fructose	Agar	56	12.00

BC biosynthesis is a complex reaction, involving different type enzyme and catalytic complexes and various kinds of protein participated in regulatory process. As mentioned above, the glucose is one of efficient and common carbon sources added into culture medium for producing cellulose. Generally, the cellulose biosynthesis process converting glucose into cellulose are separated into four enzymatic steps (see Figure 4, adapted from Lee KY et al. 2014) At first step, the glucose is phosphorylated into glucose-6-phosphate (Glc-6-P) by glucokinase, and then the phosphorglucomutase continue to catalyze the isomerization of Glc-6-P into glucose-1-phosphate (Glc-1-P), after that the uridine diphosphate glucose (UDP-glucose) is synthesized from Glc-1-P through the catalytic of UDP-glucose pyrophosphorylase (Ross et al. 1991). Finally, the UDP-glucose (UDPGlc) is able to be as substrate to add the glucose into cellulose by cellulose synthase (Ross et al. 1991). But the cellulose synthase must be activated by the cyclic diguanylate (c-di-GMP). Once the attendance of c-di-GMP in cellulose biosynthesis process, it would make the activity of cellulose synthase increases up to 50 – 100 times than before (Brueau and brown 1987). In other words, the presence of the c-di-GMP plays a significant role in increasing BC productivity. While, c-di-gmp is made through diguanylate cyclase and degraded by phosphodiesterases (Ta et al. 1998). The Last step, the UDPGlc is polymerized into cellulose by cellulose synthase (Ta et al. 1998). In addition, the UDPGlc is another important precursor in synthesis of cellulose process and also commonly exists in many organisms (Ta et al. 1998). The UGPase actives in cellulose producer when doing cellulose synthesis, it is up to hundred times more active than other bacteria without cellulose synthesis function (Valla S et al. 1989). Refer to recent literature, it is well studied as shown in later figure 4.

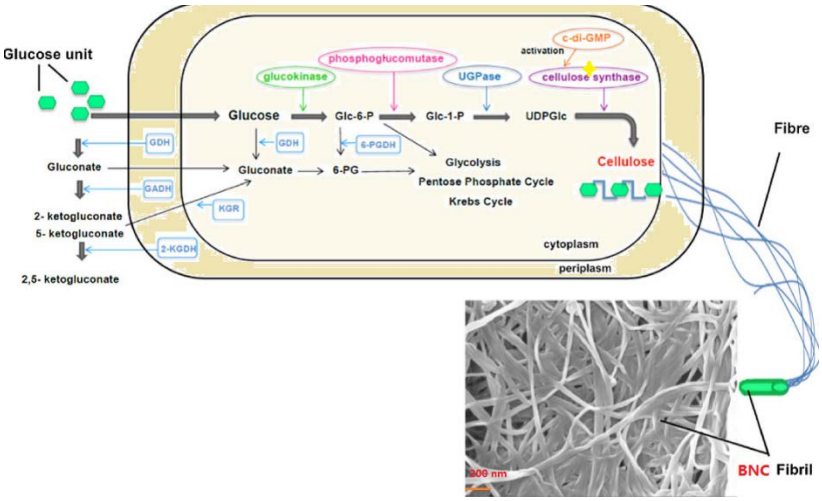


Figure 4. *A schematic shows a major metabolic pathway of K. xylinum and cellulose molecules assembled into nanofibrils process (adapted from Lee KY et al.2014).*

As can be seen in Figure 4, BC is found to be formed between the outer and cytoplasm membranes of the cell. Cellulose is synthesized in microorganisms in two intermediary steps. First step, the 1, 4-beta-glucan chains are formed. Second step, cellulose chains are assembled and crystallized into micro fibrils. And assembly and crystallization of cellulose is important and rate-limiting step (Deiannino et al. 1988, cited in Lee KY et al. 2014). Each single cell of *Komagataeibacter* has a lineal pore rows, where the cellulose ribbons formed by micro fibrils can be secreted through these pores from periplasm membrane into culture medium successfully (Deiannino et al. 1988, cited in Lee KY et al. 2014). The formation process of ribbon assembly in the *K. xylinum* bacteria system is showed in Figure 5.

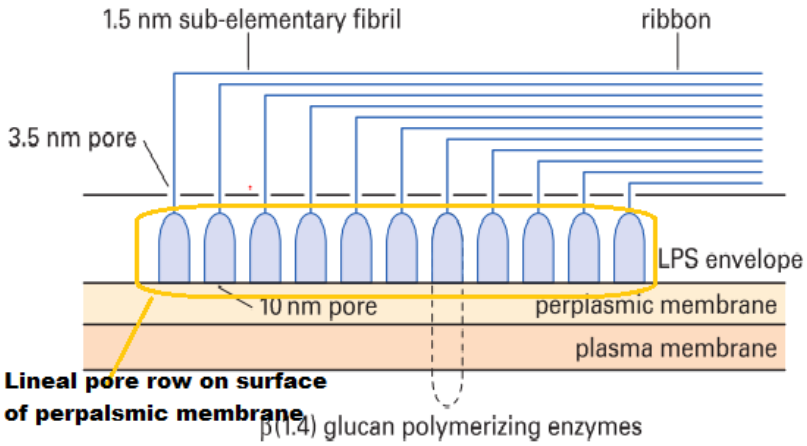


Figure 5. *The diagram model represents the formation process of ribbon assembly in the Acetobacter xylinum bacteria system (adapted from Vandamme et al. 1997).*

Furthermore, if the carbon sources utilized in cellulose biosynthesis are the disaccharides such as sucrose and maltose, the disaccharides must be hydrolyzed into monosaccharides at the beginning of the BC biosynthesis process (Jozala AF et al. 2106). Importantly, Kuo

et al. (2015) reported that the glucose is the most often used carbon sources because of it is both in energy sources and precursor for obtaining BC during biosynthesis process. Even though, the phenomena of low BC yield often occurred because of the presence of glucose dehydrogenase, which convert glucose into gluconic acid, hence decreasing the pH of culture medium and influence the BC biosynthesis (Kuo et al. 2015). Thus, in order to address this limitation, Kuo CH et al. 2015) generated a mutant *K. xylinus* strains by knocking out the membrane bound glucose dehydrogenase (GDH) gene via homologous recombination of a defect GDH gene. Since the GDH locates in the cytoplasmic membrane of *K. xylinus* and oxidizes glucose to gluconic acid that decreases the glucose conversion to BC production (Kuo CH et al. 2015). Thus, the results of Kuo CH et al. (2015) showed that, without membrane bound GDH, the mutant strains still be able to produce BC, it increased the glucose utilization efficiency for BC biosynthesis and obtained almost 2.3- fold higher BC yield than that of wild type strains (Kuo CH et al. 2015). In this study, we point out another reasonable strategy to address the gluconic acid inhibition effect. The cellulose producer is attempted to co-cultivate with other bacteria strain (*A. baylyi ADP1* Δ*gcd*) having capability of utilizing gluconic acid but without consuming glucose function. This strategy will reduce the amount of gluconic acid produced to maintain pH of cultivation at optimized range in cellulose productivity process, and then enhances BC production.

II. Genetic pathway of BC biosynthesis (Komagataeibacter geneus)

Through the genetic pathway, the BC is synthesized and regulated by the cellulose synthesis operon including four genes- bcsA, bcsB, bcsC, and bcsD. Each of these four genes plays different roles in the BC synthesis process. The bcsA- the first gene in the bcs operon, encodes a single polypeptide- the catalytic subunit of cellulose synthase, and binds to the UDPglc. And the second gene is bcsB, it plays a significant role in regulating cellulose synthase subunit to bind to c-di-GMP (Wong HC et al. 1990). This is an important step, because the cdi-GMP does affect the final BC yield indirectly by activating the bcs operon (Wong HC et al. 1990). And the cellulose fibrils are crystalized and secreted to extracellular matrix by bcsD and bcsC, respectively (Wong HC et al. 1990). The cellulose production will be reduced about 40% compared with that of wild type, if the bcsD was blocked (Wong HC et al. 1990). The reason is that the bcsD could be expected to participate in producing one product, which is localized on the periplasm membrane, and this product is responsible for guiding the glucan chains exported through the pores into culture medium (Wong HC et al. 1990). From *Figure 6*, it shows how the operon take part in the BC biosynthesis in a cell of bacterium (using different color marked these operon genes).

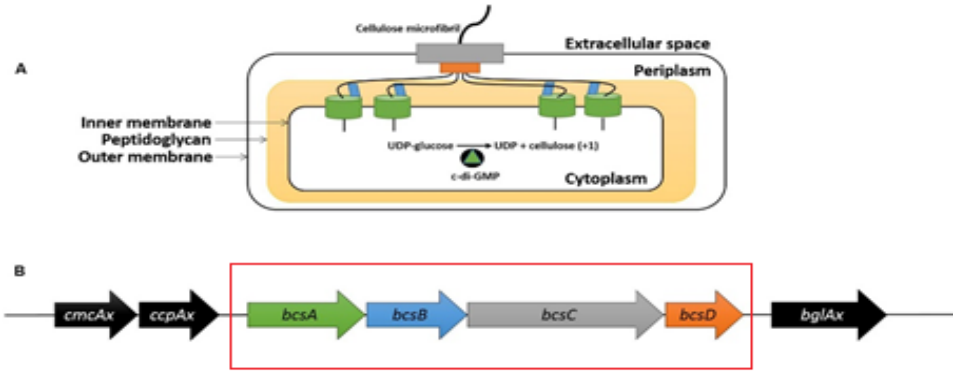


Figure 6. *Structural and genetic organization of the BC biosynthesis complex (A) and operon genes (B). BcsA (green), activated by c-di-GMP, adds a glucose unit to the cellulose chain using UDP-glucose as a substrate in the cytoplasm; BcsB (blue) guides the glucan chain through the periplasm; BcsD (orange) crystallizes four glucan chains in the periplasm; BcsC (gray) exports the BC microfibril into the extracellular space (A). The genetic organization of the bcs operon that encodes the BC synthesis complex and the genes up- and downstream (B). The genes in the bcs operon (B) are color coordinated with their protein products (A) (adapted from Augimeri RV and Strap JL 2015).*

2.2.4 Factors influencing BC production

BC productivity could be affected by many factors, such as bacteria strains, fermentation process, pH, cultivation temperature, culture medium composition, and cultivation time and so on (Lee KY et al. 2014).

I. Genetic modified bacterium strains

BC producer (bacterium strains) is one of significant factors influencing BC production. Thus, many studies focus on the modification of bacteria strains to affect the BC biosynthesis process and then to improve the BC yield (Lee KY et al. 2014). Overexpression and disruption mutant are usually used in modifying strains technology reported in some of publications, and this final aim is to increase the BC yield with low cost by this genetic modification (Yasutake et al. 2006). As so far, there are countless success examples to get recombinant strains for producing BC, e.g., one strain of *K. xylinus* achieved a significant capacity after genetic modification, it was able to yield 28 times more BC than that of wild type strain by utilizing lactose as carbon source (Souza et al. 2014). Table 3 lists some strains from *K. xylinus* genus that have been modified by disrupting certain genes, Finally, BC production are improved due to certain functions obtained.

Table 3.*The list of Modified bacteria strains from K. xylinus genus by disrupting certain genes, and shows the achievements of effect on BC biosynthesis (adapted from Gama MD et al. 2016)*

Vectors	**Marker gene**	**Wild type**	**Genetic modification**	
			Disruption gene	**Effect on the cellulose biosynthesis**
pHSG399	*Kan*	*K. xylinus BPR2001*	*dgc1* encoding diguanylace cyclase	Weak effect on BC productivity despite strong impact on its structure.
pHSG399	*amp*	*K. xylinus BPR2001*	*omcAx* encoding the endo-β-1,4-glucanase	Altered cellulose crystallinity and morphology of the fibers as well as reducing cellulose synthesis yield
pHSG399	*amp*	*K. xylinus BPR2001*	*cpcAx* encoding the cellulose complementing factor	Affected productivity and crystallinity of cellulose.

The *K. Xylinus* is also known as *A. xylinum* or *G. xylinus* (Pecoraro et al. 2007; Kumbhar et al. 2015; Tsouko et al. 2015). Some strains of this bacterium are attempted to be isolated from fruit or food by some researchers and modified by genetic technology. The mainly purpose of this action is to find or create one strains which has capability of high BC production. The *K. xylinus* is the most popular and common bacteria, because it has high BC yield ability, and *K. xylinus* is able to produce cellulose at aerobic condition, which is non-photosynthetic process (Kongruang S 2008). And *K. xylinus* has specific ability to consume carbohydrates (e.g. glucose) and ethanol, oxidize them into gluconic acid and acetic acid, respectively, due to its aerobic metabolism (Kongruang S 2008). But, the acid formation accumulation results in the pH of cultivation dropping down rapidly. This phenomenon, not only influences the BC yield, but also inhibits the cells growth to some extent. Even though there is still no any efficient method to address this inhibition effect so far, thus, it is necessary to understanding the metabolism process when they are producing BC, which could help to find good solution to solve this problem. In this study, *K. xylinus* DSM 2325 wild type was purchased from the DSMZ, Germany. Other *K. xylinus* engineered type including pA, pAB and pABCD (see Mangyil et al. in press) are obtained in our laboratory. In this study, these wild type and engineered type were investigated for BC production. In addition, a reasonable hypothesis reported by some authors try to explain the phenomena of producing BC. As mentioned above, the BC produced by the *K. xylinus*, could be induced by some stress conditions such as low humidity, high UV light, nutrient limitation or less liquid and so on (Huang et al. 2014b). As mentioned above, Kuo CH et al. (2015) reported that the mutant of *K. xylinus* bacteria was able to yield

more BC than wild strains because of the membrane bound GDH being inactive by knocking out of glucose dehydrogenase gene (GDH) from wild strain.

II. Parameters affecting the fermentation process

The culturing condition, such as culture medium composition, pH, temperature, the fraction of oxygen supplied, static or shaking reactor used, does influence the BC productivity. Change of culturing condition could result in producing different composition, morphology and properties of BC (Hu et al. 2014).

Culture medium

Culture medium is one of important factors of BC production, it can significantly affect the final BC yield and total cost. Low cost culture medium like wastewater or waste juices was reported to be used to produce BC (Lee KY et al. 2014). In this study, MA/9 medium, a simple components and low cost culture medium, was investigated to produce BC. In addition, the composition of culture medium, not only influence BC productivity and cells growth, but also affect the pore size, polymerization degree, crystallinity, width of BC fibrils and mechanism property of BC (Tokoh et al. 1998).

In previous publications, there are various carbon sources being investigated, aiming to find the best carbon sources utilized by different bacteria strains to achieve high BC yield with low cost. Mikkelsen D et al. (2009) compared the effects of six different carbon sources like glycerol, glucose, mannitol, fructose, sucrose and galactose on producing BC by G. xylinus ATCC 53524. After incubating 4 days, even though the results demonstrated that the sucrose obtained the most amount of BC production at the end of fermentation, and the glycerol, mannitol, glucose and fructose were ranked from the second to the sixth, respectively (Mikkelsen D et al. 2009). But, at the initial incubation time (84 h), the galactose and sucrose only got first and second lowest BC production, respectively, because the sucrose was unable to be utilized directly and it must be hydrolysed into glucose or fructose at first in the periplasm (Ross et al. 1991). While, the galactose was difficult to be transported into cell membrane, it means that it was hard to be uptake from culture medium by BC producer (Velasco-Bedran and Lopez-Isunza 2007). Those reasons result in the lower BC yield compared with that of other four type carbon sources (Mikkelsen D et al. 2009). In Mikkelsen D et al. (2009) investigation, he also found that the crystallinity degree of BC utilized this six carbon sources was observed to be similar (up to 80 and 90%). The D-mannitol and D-atabitol has been reported to be the best carbon sources, since they were able to produce approximately 6.2 and 3.8 times more cellulose than other carbon sources, respectively (Joans and Farah, 1998), even though the price of them are still very high. And in Coban and Biyik investigated results, it demonstrated that the BC productivity of *A. Lovaniensis* HBB5 was increased by supplying yeast extract and glucose into HS medium (Coban et al. 2011).

Masaoka S et al. (1993) reported that the BC yield used glucose as carbon source was approximately 10 folds more than that from maltose containing culture medium. Therefore, in previous publication, the glucose was widely utilized in producing BC production. However, a generated problem is that gluconic acid was formed as product after glucose being consumed by bacteria in BC biosynthesis process. Hence, the gluconate was becoming the significant parameter effect of the BC productivity by decreasing the pH of culture medium. In addition, it was known that the utilized glucose equals to the total BC yield and gluconic acid amount. The redundant glucose could be metabolized into gluconic acid, once the glucose was supplied too much to utilize for producing BC. Further, it was found that the optimized glucose concentration for BC production was around 1.5% to 2%. The amount of BC production could be decreased if the glucose concentration increased above 2% (Keshk et al. 2006). It was also proved that the amount of gluconic acid increased and BC yield decreased with the initial high glucose concentration added in culture medium (Masaoka et al. 1993).

Except the carbon sources, the nitrogen sources also play a significant role in producing BC. Since nitrogen sources can supply amino acid, vitamins and mineral salts for bacteria growth and BC yield. In HS medium, the main and basic component are yeast extract and peptone, which are considered to be most popular and best nitrogen sources for BC producer. According to previous publications, it was found that there were many studies focusing on the various nitrogen sources effect of BC production. Matsuoka et al. reported that supplying extra nitrogen sources addition into culture medium was able to enhance BC yield and increase bacteria population (Matsuoka et al.1996).

Besides, Park et al. (2003) pointed out that the ethanol addition in culture medium was beneficial to increase BC production. Because the ethanol can prevent the BC producer from mutating by itself into bacteria lost cellulose producing function. Shigemtsu T et al. (2005) pointed out that ethanol supplementation plays a role in the energy supply for ATP generation, which could enhance GHK activity, resulting in improved glucose metabolism for BC production. Glycerol has been reported as carbon sources for BC production by *Komagataeibacter* strains, even though the BC yield from glycerol containing culture medium was lower than that from glucose in static culturing condition. In contrast, in shaking culturing condition, the BC yield from culture medium contain glycerol was up to 2.16 g/L, much more than that from glucose containing medium (Jung JY et al. 2005).

pH

The optimum pH of culture medium used in BC production is determined by the particular bacteria strains. Generally, when the pH of culture medium is in range of 4.5 to 7.5, BC production was able to be produced more than that of other pH range, specifically, Son HJ et al. (2001) pointed out that highest BC yield was obtained when pH at the 6.5.

The pH of culture medium usually decreased by the some secondary metabolism product accumulation, such as gluconic acid, acetic acid or lactic acid and so on (Zeng XB et al. 2011). Those secondary metabolites were formed by carbon sources and nitrogen sources consumption process for producing BC production (Zeng XB et al. 2011). Therefore, maintaining pH of culture medium could be beneficial to obtain high BC yield.

Temperature

The temperature of incubation not only affects the BC yield, but also influences the morphology and structure of BC. Hirai et al. (1997) mentioned that, the cellulose produced in HS medium at low temperature (4 ℃) was band shaped with cellulose Ⅱ structure while produced at 28 ℃, the structure of cellulose was changed into cellubse Ⅰ ribbons (Hirai et al. 1997). In addition, the BC yield decreased with the increasing of temperature above 35 ℃(Wong HC et al. 1990). Interestingly, compared with 30 ℃, the BC yield did not reduced when the temperature was decreased to 25 ℃ (Wong HC et al. 1990). Son HJ et al. (2001) found that the optimum temperature of incubation for BC production was 30 ℃.

Oxygen

Shirai A (1994) pointed out that the dissolved content of oxygen influenced not only the growth of cells but also the BC yield and BC quality (Shirai 1994, cited in Lee K et al. 2014). Specifically, concentration of gluconic acid increased with the high dissolved content of oxygen in culture medium (Tantratian et al. 2005). And then, the cells growth would be affected by the high concentration of gluconic acid in culture medium, which would result in low BC yield at same time. While, the low dissolve content of oxygen also inhibit the respiration metabolism of cells and then result in low yield of BC (Tantratian et al. 2005). Thus, Hwang JW et al. (1999) reported that the optimum dissolved content of oxygen was 10%, thus, at this range of oxygen content, bacteria was able to produce maximum amount of BC (Hwang JW et al. 1999, cited in Lee KY et al. 2014).

Bioreactors and cultivation time

Static and shaking culture are commonly two method for producing BC, which culture method should be chosen depend on the BC final application, because the different culture methods determine the morphological, mechanical and physical properties and cellulose production (Lee KY et al. 2014). Horning M et al. (2009) reported that the shake cultures can be used in order to reduce the cultivation period, and the chemical structure of BC produced in static and shaken condition is identical. Importantly, Dudaman WF et al. (1960) mentioned that the culture time of static condition (3-4 weeks) can be reduced to 2-4 days under the shaken condition. In recent publication, the formation of BC increased with the increasing of growth time (Sheykhnazari et al. 2011).

2.2.5 Potential application of BC

The most popular of BC application in food industry is the Nata- a traditional dessert and fermented by the *K. xylinus* bacterial. The *Table 4* shows the potential application of BC in different area in prevent few years.

Table 4. *The BC application in different area (adapted from Jozala AF et al. 2016).*

Application area	**BC application**
Cosmetics	Stabilizer of emulsions like creams, tonics, conditioners, nail polished
Textile industry	Sports clothing, tents and camping equipment
Mining & refinery	Sponges to collect leaking oil, materials for absorbing toxins.
Waste treatment	Recycling of minerals and oils
Sewage purification	Urban sewage purification, ultra-filtration water
Communications	Diaphragms for microphones and stereo headphones
Food industry	Edible cellulose (nata de coco)
Paper industry	Artificial replacement of wood, special papers
Medicine/biomedical	Temporary artificial skin for burns and ulcers, dental implant components; Antimicrobial wound dressing, Nanofilm, Drug Delivery, Drug excipient
Laboratories	Protein immobilization, chromatographic techniques, tissue culture medium
Electronics	Optoelectronics materials (liquid crystal displays)
Energy	Membrane fuel cell (palladium)

In paper industry, the BC can be used for producing one carbon paper based on the electrically conductive property of BC (Miyajima et al. 2016), and this carbon paper are often utilized in fuel cells. The Barud and Mautner (2015) also pointed out that the BC can be used to develop bio-cellulose flexible magnetic paper and nanopaper, based on the magnetic and nanofiber characteristic of BC, respectively (Barud et al. 2015). This product is known as the magnetic BC hybrid, it is often applied in purification and filtration area, such as loudspeaker membrane and toxic wastewater treatment and so on (Barud et al. 2015). Those applications have un-imaginative commercial value and address many urgent problems in various industries. That is the reason why the BC can attract many scientist attentions.

In healthcare industry, BC can be used in skin regeneration field such as artificial skin, due to its non-toxic mature, easy to handle, modify its surface properties and environmental treating abilities (Yang HS et al. 2004). In cosmetics industry, BC also has increasingly applications. The cosmetics, which is defined as a "product" applied to human body for cleansing, beautifying, promote the attractiveness and change the appearance without affecting the body structure or functions" (Hasan et al. 2012). Since most of materials from cosmetics contacting with skin will cause some problems, such as irritation reaction and skin allergies. Therefore, BC is used as natural skin preferred cosmetics materials, required and having huge marketing demand in recent few years. Besides that, BC as green biomaterials, also has spread commercial applicability, mentioned in present published articles (Yang HS et al. 2004), shown in Table 5.

Table 5. *Some BC as green materials applied in many industries mentioned in previous peer-review articles (adapted from Gallegos et al. 2016).*

BC-based materials	Methodology	New functionalities	Potential application	Reference
BC/Chi/Alg or BC-Vaccarin	Molding Immersion	Physical, mechanical, biocompatibility	Wound dressing	Chang & Chen 2016; Qiu et al. 2016
BC-xGnP	Impregnation	Thermal properties, electrical conductivity	Biosensors, tissue engineering	Kiziltas et al. 2016
BC-Fe_2O_3	Immersion	Magnetic behavior	Magnetic paper, loudspeaker	Barud et al. 2015
BC-HA	Immersion	Biocompatibility	Bone tissue regeneration	Duarte et al. 2015
AMPS-g-BC	Ultraviolet-induced polymerization	Conductivity, effective methanol barrier	Fuel cells	Lin et al. 2013
ε-PL/BC	Immersion	Physical, antibacterial	Packaging	Zhu et al. 2010

2.3 Acinetobacter baylyi ADP1 Δgcd

2.3.1 Description of *A. baylyi ADP1* Δgcd

A. baylyi ADP1 is a common model bacterium due to its capability of being engineered easily and utilizing wide substance (Myllyntausta S et al. 2009). Glucose is the only sugar,

and *A. baylyi ADP1* can consume and catabolize glucose into gluconate at first step by glucose dehydrogenase (Barbe V et al. 2004). In Kannisto et al. publication, the glucose metabolism of *A. baylyi ADP1* was disabled by knocked out gcd gene from its genome for glucose dehydrogenase. In Figure 7, it showed the gcd knockout steps and their sites for restriction enzyme, and in (b), the PCR verification bands were showed including wild type and gcd knockout out engineered type of *A. baylyi ADP1*(2015).

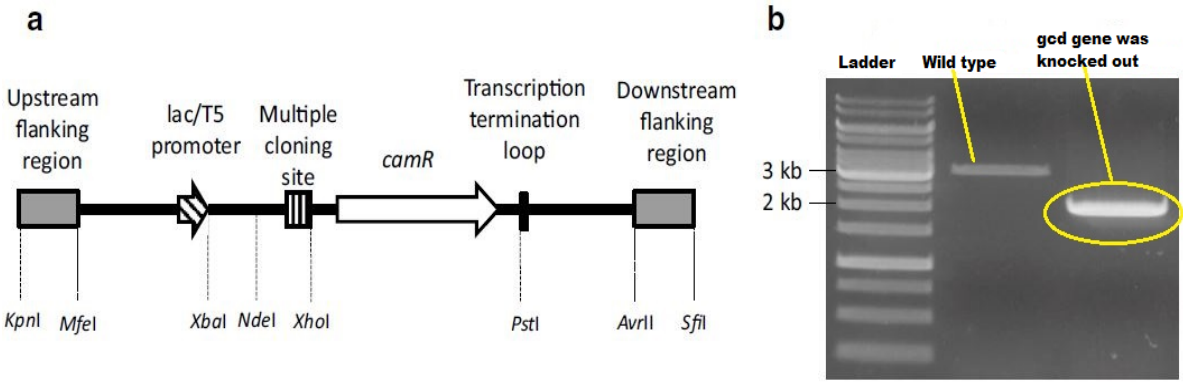


Figure 7*. Simplified representation of gcd cassette (a) and PCR verification of transformation (b) (Adapted from Kannisto et al. 2015).*

Moreover, from the results of Kannisto et al. (2005) they also proved that the engineered strain- *A. baylyi ADP1* Δgcd was unable to utilized sugars like glucose, arabinose and xylose, but, it was capable of consuming gluconate well and rapidly.

2.3.2 Carbon catabolite pathway of *A. baylyi ADP1 Δgcd*

From Figure 8, it shows the change of glucose metabolism of *A. baylyi ADP1* after removing the gcd gene, and it is interesting to be noted that the step of glucose converting into gluconate was blocked due to gcd gene disappeared.

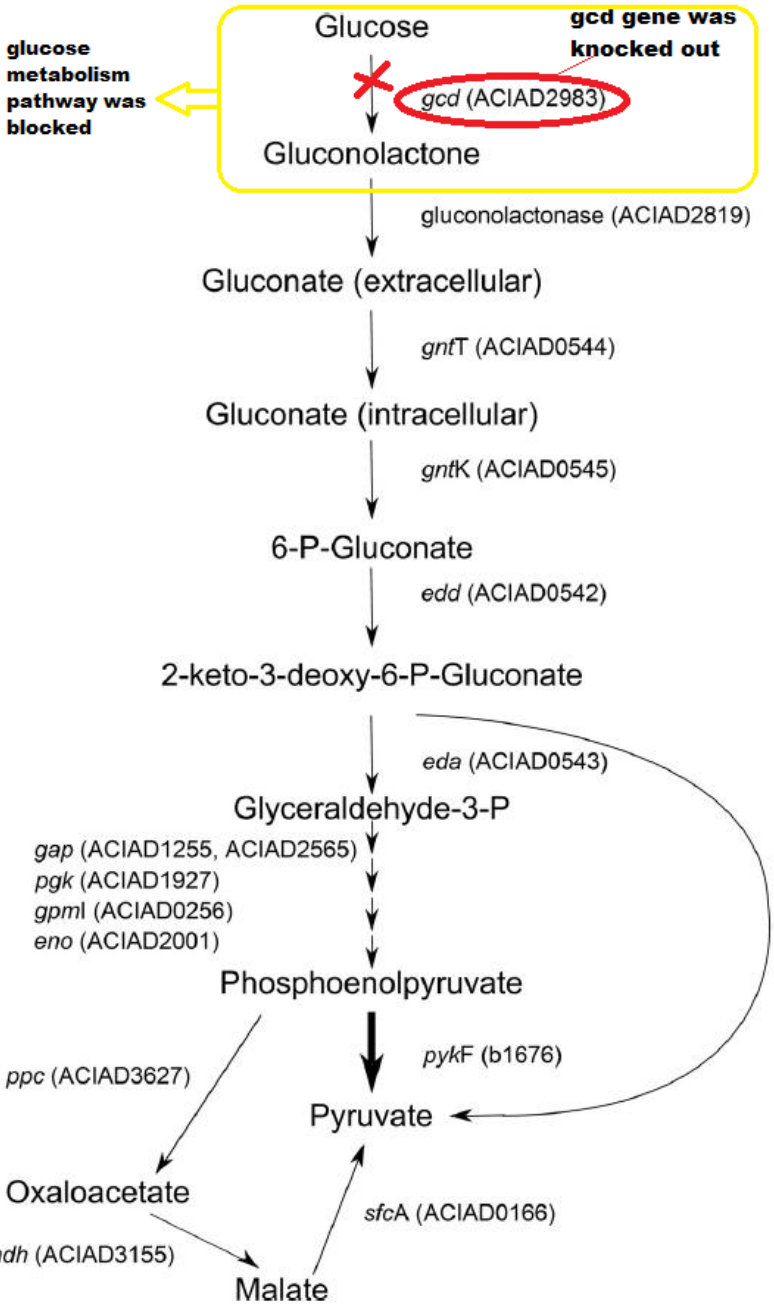


Figure 8. *It shows the change of A. baylyi ADP1 carbon sources metabolism pathway after knocking out gcd gene (Adapted from Young DM et al. 2005).*

This mutant make the *A. baylyi ADP1* to utilize gluconate directly without glucose dehydrogenase step. The purpose of removing gcd gene from wild type of *A. baylyi ADP1*, is that the *A. baylyi ADP1* growth on glucose is quite slow, and it must catabolizes glucose with Entner-Doudoroff pathway (Figure 8). In wild type of *A. baylyi ADP1* pathway, the glucose molecule must be converted into gluconate, and then the gluconate was continually converted into pyruvate molecule and glyceraldehyde-3-phosphate. Although the former can produce pyruvate directly in citric acid cycle. The latter cannot be converted to pyruvate directly (Dolin MI and Juni E 1978), if it is to be oxidized to CO_2. The key point

is that the *A. baylyi ADP1* lacks one gene involving phosphoenolpyruvate-pyruvate-oxaloacetate node's common enzymes and pyruvate kinase expression. Thus, Kannisto M. engineered *A. baylyi ADP1* to express pykF gene for pyruvate kinase, which enhanced the gluconate metabolism of *A. baylyi ADP1* compared with that of wild type (2014).

The results of Kannisto M et al. (2014) indicated that engineered cells of *A. baylyi ADP1* grew 2-fold faster with increasing gluconate metabolism than wild type. And the high growth rate of engineered cells of *A. baylyi ADP1* also was not accompanied with reduction of wax ester production. The engineered strain- *A. baylyi ADP1 Δgcd* was able to grow fast by utilizing gluconate directly and rapidly, but it loss the function of using glucose by removing gcd gene, which has been reported by Kannisto et al. (2015). That is the reason why *A. baylyi ADP1* Δgcd can be used for co-cultivating with *K. xylinus* for BC production in this study. Because it has capability of consuming gluconate for its growth but not utilizing glucose, so it might be able to address the gluconate accumulation in BC production of *K. xylinus* on culture medium. It might benefit to achieve the goal of high BC yield.

3. METHODOLOGY AND MATERIALS

Although for BC production process, many new complex bioreactors have been developed yet, the static cultivation are still preferred. And the large surface area plays significant role in good productivity (Jonas R and Luiz LF 1998). Hence, in this thesis study, the square petri dish, which has large surface area, was used as culture dish for BC production. And all incubating condition of BC production experiment was at 30 ℃ static condition.

3.1 Bacterial Strains

Bacterial strains *K. xylinus* DSM 2325 (WT) was purchased from DSMZ Company (DSMZ, Germany). And the recombinant of *K. xylinus* strains (pABCD) were constructed by cloning *K. xylinus* bcs operon genes (bcsA, bcsB, bcsC and bcsD) to pBAV1C vectors, the pABCD strains contains CAM resistance gene and arabinose promoter (Mangyil et al. In press).

The *A. baylyi ADP1* DSM 24193 also was purchased from DSMZ, Germany. The engineered type from which *A. baylyi ADP1 Δgcd* was constructed by knocking out the gcd gene (Kannisto et al. 2015).

3.2 Medium compositions and reagents.

Hestrin and Schramm medium (HS medium) was used for precultivation and BC production experiment. The components included 5 g/L peptone (Lab M Limited, UK), 5 g/L yeast extract (Fisher Scientific, UK), 2.7 g/L di-sodium hydrogen phosphate (Na_2HPO_4) (VWR prolabo chemicals, EU), 1.15 g/L citric acid (EMD Millipore corporation, Germany). And the HS medium pH was adjusted to 6.0 with 0.5 M HCl. And 2% (w/v) D-glucose (Amresco-inc, US) was added into medium as carbon sources when used for precultivation of wild type and engineered type of *K. xylinus*. Both HS and MA/9 medium were supplemented with 250 μg/mL CAM (only for recombinant *K. xylinus* pABCD) and 0.2% (v/v) cellulase for precultivation and growth experiment.

Seed inoculum for experiments with *K. xylinus* were prepared by streaking the glycerol stocks on HS agar plate added15 g/L agar (Lab M Limited, UK). The plates were incubated at 30 ℃ for 3 days. Single clones were inoculated into 5 ml HS medium containing glucose, 0.2% (v/v) cellulase and 250 μg/mL CAM (for recombinant strains) and grown at 30℃/180 rpm. To achieve high optical density (OD 600 nm), the grown cells were further inoculated to 45 ml of similar growth medium.

MA/9 minimum medium was used in the growth and BC production experiments. The components include 5.52 g/L di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (VWR chemicals, EC), 3.4 g/L potassium hydrogen phosphate (KH_2PO_4) (Malinckrodt Baker B.V, Holland), 0.96 g/L ammonium chloride (NH_4Cl) (Merck KGaA, Germany), 0.008 g/L nitrilotriacetic acid (Acros, Germany), 1g/L sodium chloride (NaCl) (EMD millipore corporation, Denmark), 0.24 g/L $MgSO_4$, 0.0111 g/L $CaCl_2$ and 0.001 g/L $FeCl_2$. Before preparation of MA/9 medium, 10 mg/ml $FeCl_2$ stock solution, 1M $MgSO_4$ stock solution and 1M $CaCl_2$ stock solution were prepared at first, it is due to precipitation of those compounds when autoclaved. Thus, those three stock solution were sterilized by being filtered through 0.22 ϕ μm disk filter. All stock solution was diluted in MQ water (Millipak, France) in this study. 2% (w/v) glucose (Amresco-inc, US) was added into MA/9 medium as carbon source in this study. The pH of MA/9 medium was adjusted to 6.8 with 0.5 M HCl.

Lysogeny broth (LB) medium was used in the precultivation of *A. baylyi ADP1* Δgcd when doing co-cultivation with *K. xylinus* for BC production and growth experiment. The components include 10g/L tryptone (Lab M Limited, UK), 5g/L yeast extract (Fisher Scientific, UK) and 5g/L sodium chloride (NaCl) (EMD millipore corporation, Denmark). 2% (w/v) glucose was used as carbon source.

For LA plate, 15 g/L agar (Lab M Limited, UK) was added, 50 μg/ml CAM was added into medium after autoclaving, the CAM was used for the screening of *A. baylyi ADP1* Δgcd transformant when doing seed culture of *A. baylyi ADP1* Δgcd.

Reagents

40% (w/v) glucose (Amresco, USA) stock solution; Trichoderma reesi ATCC 26921 cellulase (Sigma-alorich, Denmark), 10% (w/v) arabinose (Acros, China) stock solution; 50 mg/ml CAM (Sigma-alorich, China) stock solution (diluted with 70% ethanol solution), 1M (mol/l) d-gluconic acid sodium and 0.5 M NaOH solution and 80% glycerol (Sigma-alorich, Malaysia). All of mediums, reagents and flasks were sterilized in autoclave (GmbH, Austria), except cellulase and 50 mg/ml CAM (stored in 4℃ freezer).

3.3 Cultivations

3.3.1 Precultivation

Wild type of *K. xylinus* DSM 2325

Wild type *K. xylinus* DSM 2325 was maintained as frozen glycerol stocks at −80 ◦C. All starter cultures were grown by inoculating in a single rough colony of *K. xylinus* from an HS agar plate streaked from glycerol stock. And it was grown in 50 ml of HS medium

supplemented with 0.2%(w/v) cellulase and 2% (w/v) sterilized glucose in 250 ml covered Erlenmeyer flask, and incubated at 30℃/180 rpm in incubator shaker (KS 4000i control, IKA, US) for 2 days. After that the precultivation was ended until the OD_{600} reached to 1.0 above by OD measurement (Utrospec 500pro, Amerisham- Biosciences, England). A control experiment without inoculating wild type *K. xylinus* DSM 2325 was performed simultaneously. All the inoculating steps are conducted in laminar flow (Kojari tech oy, Finland).

Engineered strains of *K. xylinus* DSM 2325

The engineered type pABCD strains of *K. xylinus* DSM 2325 was also stored as frozen glycerol stocks at −80 ◦C. The cultivation of engineered strains was similar to that of Wild type *K. xylinus* DSM 2325 mentioned above. The difference was that 250 μg/ml CAM should be added into culture medium of engineered strains of *K. xylinus.*

***A. baylyi ADP1* Δgcd**

The engineered type *A. baylyi ADP1* Δgcd was also stored at −80 ◦C. A single rough colony of *K. xylinus* from an LA plate (supplemented 50 μg/ml CAM) was inoculated into 50 ml of LB medium (added 50 μg/ml CAM) contained in 250 ml covered Erlenmeyer flask. It was incubated at 30 ℃ and 180 rpm in incubator shaker (KS 4000i control, IKA, US) overnight. A control experiment without inoculating *A. baylyi ADP1* Δgcd strains was performed at same time.

3.3.2 Comparison of cells growth and BC production between *K. xylinus* WT and pABCD cultivated in different culture medium

Growth test

The wild type (WT) and engineered type (pABCD) of *K. xylinus* DSM 2325 were precultivated at 30 ℃/180 rpm, for 2 days. And those precultivated strains were inoculated in two different culture medium including 50 ml HS medium and 50 ml MA/9 minimum medium, both culture medium was added 2% (w/v) glucose and 0.2% (v/v) cellulase. Growth medium of engineered strains were extra supplemented 250 μg/ml CAM. The experiment were conducted in triplicates. The control experiment was also prepared. The initial OD_{600} of all inoculum in was 0.02. The growth cultivations in HS culture medium were incubated at 30 ℃/180 rpm, in incubator shaker (KS 4000i control, IKA, US) for 4 days and 5 days, respectively. OD measurement was carried out daily. At the end of cultivation, pH value of each sample was measured.

BC production test

Similar to growth experiment, the pABCD and WT strains were carried out BC production experiment. All of those precultivated WT and pABCD engineered strains were inoculated into 50 ml/100 ml sterilized square petri dish contained MA/9 medium supplemented with 2% glucose and 1% arabinose. The initial OD_{600} inoculant was 0.02. And 250 μg/ml CAM was added into HS and MA/9 culture medium of pABCD strains. The experiment were conducted in triplicates. A control experiment (without inoculating strains) was performed simultaneously. All cultivations of BC production are incubated at 30 °C, 180 rpm static condition for 5 days in incubator (Teimaks B8133, Norway). 1ml initial sample at 0 hour and final sample after 5 days was taken and analyzed by HPLC analysis method. After 5 days, BC sheet treatment and analysis was carried out. The dried BC sheets weight yielded were weighed by scale.

3.3.3 Optimization of glucose concentration for BC production.

Growth test

The growths of pABCD were compared by cultivations with different concentration of glucose in MA/9 medium. The glucose with certain concentrations used in this experiment are shown as follows: 0%, 0.5%, 1.5%, 2%, 3.5% and 5%. Cultivations were carried out in 50 ml/ 250 ml sterilized and covered Erlenmeyer flask contained MA/9 medium added certain concentration of glucose mentioned above. The experiment were conducted in triplicates. In addition, before inoculation step, all of MA/9 medium were added 50 μg/ml CAM and 0.2% (v/v) cellulase. The engineered type pABCD strain precultivated for 2 days were used as inoculant. And the initial inoculum OD_{600} was 0.02 and the strains were cultivated at 30 °C/180 rpm, for 5 days in incubator shaker. A control experiment without inoculating pABCD strains of *K. xylinus* DSM 2325 was performed simultaneously. OD measurement was carried out 3 times per day. The pH measurement was carried out to each sample at the end of cultivation.

BC production test

The BC production of engineered type pABCD of *K. xylinus* DSM 2325 were also compared by cultivations with 0%, 0.5%, 1.5%, 2%, 3.5% and 5% glucose in MA/9 medium. All the cultivations were carried out in 50 ml/100 ml sterilized square petri dish contained MA/9 medium, and certain concentration glucose, 50 μg/ml CAM and 1% (w/v) arabinose was supplemented in MA/9 medium. The experiment were conducted in triplicates. The precultivated pABCD strains were inoculated into each sample medium with 0.02 initial OD_{600}. A control experiment without inoculating pABCD strains was performed simultaneously. All of BC production cultivation are incubated at 30 °C, static condition for 5 days in incubator (Teimaks B8133, Norway). Taking 1ml initial sample at 0 hour and final sample at 5 days for HPLC analysis. After 5 days, BC sheet treatment was carried out and weighed.

3.3.4 The effect of arabinose on BC production of pABCD engineered strains in MA/9 medium.

Similar to BC production experiment mentioned above, the precultivated pABCD strains were inoculated in MA/9 medium supplemented with 2% (w/v) glucose and 250 μg/ml CAM. Four group samples were prepared, supplemented 0%, 1% arabinose into MA/9 culture medium, inoculated 0.02 and 0.04 initial OD_{600} of pABCD for BC production, respectively. All cultivations for BC production were carried out in 50 ml/100 ml sterilized square petri dish (Greiner bio-one, Hungary), and all petri dishes were put into covered plastic box (size: 34 cm*25 cm*16 cm) (Othexgroup, Sweden). Two full bottles MQ water was put insides of plastic box when doing incubation. After that, this plastic box was placed in incubator (Teimaks B8133, Norway) and incubated at 30 ℃, 180 rpm static condition for 5 days. The size of petri dish was 120mm*120mm*17mm. 1ml initial sample at 0 hour and final sample at 5 days were taken for HPLC analysis. After 5 days, BC sheet treatment and analysis was carried out. The dried BC sheets weight yielded were weighed by scale.

3.3.5 Testing the concentration of gluconate which utilized by *A. baylyi ADP1* Δgcd.

The precultivated *A. baylyi ADP1* Δgcd strains was inoculated in MA/9 medium supplemented 50 μg/ml CAM and different concentration of gluconic acid sodium, the certain concentration used in this experiment are shown as follow (mM=mmol/L): 0 mM, 10 mM, 30 mM, 50 mM, 80 mM, 110 mM and 200 mM gluconic acid sodium. Triplicate was performed for each sample. And a control experiment was also prepared without inoculating any strains. All of cultivation for this experiment were incubated at static 30 ℃, 180 rpm condition, in incubator shaker for 2 days. The pH and OD measurement was carried out 3 times daily, 1ml sample from each flask was taken and stored in Eppendorf tube at - 50 ℃ for HPLC analysis.

3.4 Co-cultivation experiment

Growth test

A single colony of pABCD of *K. xylinus* DSM 2325 taken from HS agar plate was precultivated in HS medium supplemented 2% glucose, 250 μg/ml CAM and 0.2% (v/v) cellulase, and incubated at 30 ℃/180 rpm for 2 days. A single colony of *A. baylyi ADP1* Δgcd taken from the LA plate was precultivated in 50 ml LB medium added 50 μg/ml CAM at 30 ℃/180 rpm in incubator shaker overnight.

Two groups of sample were prepared, i.e., co-cultivation group, no co-cultivation group.

The MA/9 medium supplemented 2% glucose, 50 μg/ml CAM and 0.2% (v/v) cellulase was used as culture medium. For co-cultivation group, 0.02 initial OD_{600} of precultivated pABCD and *A. baylyi ADP1* Δgcd strains were inoculated into the 50 ml/250 ml covered Erlenmeyer flask containing MA/9 medium at same time. For no co-cultivation group, the precultivated pABCD strains was inoculated alone into 50 ml same MA/9 medium as co-cultivations. The experiment were conducted in triplicates. *A. baylyi ADP1* Δgcd strains also was inoculated into MA/9 medium alone as no co-cultivation group, even though, it could not grow well, because it was unable to utilize glucose as carbon sources added in this MA/9 medium. A control experiment without inoculating any strains was also prepared. All of cultivations were incubated at at 30 ℃/180 rpm in incubator shaker for 5 days. OD measurement was carried out 3-4 times per day. 1 ml sample from each flask was taken and stored at - 50℃ for HPLC analysis.The pH measurement was carried out to each sample at the end of cultivation.

BC production test

Similar to growth test of co-cultivation mentioned above. In the co-cultivation group, *A. baylyi ADP1* Δgcd and pABCD was incubated in 50 ml/100ml square petri dish. Each of petri dish was filled in 50 ml MA/9 medium containing 2% (w/v) glucose, arabinose (1%) and 50 μg/ml CAM. The initial OD_{600} of *A. baylyi ADP1* Δgcd and pABCD was 0.02. No co-cultivation group only inoculated pABCD alone in same MA/9 medium. The experiment were conducted in triplicates. Two control group were prepared at same time, one was blank I group without inoculating any strains, the other one was blank 2 group only inoculating *A. baylyi ADP1* Δgcd alone. All of BC production samples in petri dishes were put into covered plastic box (size: 34 cm*25 cm*16 cm) (Othexgroup, Sweden). In order to prevent the evaporation of growth medium, a beaker containing water was placed in the plastic box. After that, this plastic box was placed in incubator (Teimaks B8133, Norway) and incubated at 30 ℃/180 rpm static condition for 5 days. 1 milliliter (mL) of culture sample at 0 hour and after 5 days were taken for HPLC analysis. BC sheet treatment and dried BC sheet of weighing were carried out.

3.5 Analysis methods

3.3.1 Optical density analysis

OD_{600} was used to determine the growth rates of cells. The interval time of measurements was 5-8 h, depending on each experiment, and the first data point from the beginning of each cultivation. Slopes of the ln (OD_{600}) versus time (in hours) were used to determine the growth rate in the exponential phase. In this study, the spectrophotometer 500 pro (Amerisham Biosciences, England) was used to measure the optical density of culture sample. Sterile ion exchanged water (MQ water) was used in diluting the cultivations

where necessary.

3.3.2 HPLC analysis

The end-products of the cultivations were measured with HPLC. Through HPLC analysis, the concentration of glucose and gluconic acid of culture sample was determined.

HPLC Sample preparation

Culture samples was thawed at room temperature and centrifuged at 12000 rpm for 5 minutes. 1ml soft-Ject syringe (Henke sass wolf, Germany) was used to collect the supernatant without disturbing the cell pellet. And the supernatant was filtered through chromafil xtra ® PET - 20/25 polycarbonate filter (VWR, Germany) and diluted 10 times with MQ water in 1.5 ml HPLC wide open vials (VWR, Germany). The pore and filter size of this syringe filter and HPLC vial were 20 μm, ϕ25 mm and 32 mm*11.6 mm, respectively.

HPLC analysis

The samples were analyzed for gluconate, glucose with LC-20AD prominence liquid chromatograph (Shimadzu, Japan) equipped with RID-10A refractive index detector (Shimadzu, USA), DGU-20A5 prominence degasser, CBM-20A prominence communications bus module, and SIL-20AC prominence autosampler. Phenanenex therm sphere (Phenanenex, USA) kept at 40 °C was used as a column. Sulfuric acid (0.01 N) was used as an eluent at pumping rate of 0.6 ml/min. Identification and quantification of liquid end products were based on co-chromatography of using standards. The concentration of glucose standard was prepared and run from 0.5 mM, 1 mM, 5 mM, 7.5 mM and 10mM, and the concentration of gluconic acid sodium standard was prepared as same as the glucose standard preparation. Blank culture mediums and MQ water were used as control groups.

3.3.2 BC sheet treatment and analysis

BC sheet was produced after several day static cultivation in incubator. The next step was to treatment the BC sheet. Firstly, the extra medium was removed from BC sheet. And the harvested BC sheet was washed with MQ water to remove extra cells and culture medium from the surface of sheet. To inactive the cells, 0.5 M NaOH solution was used to wash sheet again, and incubated sheet in 0.5 M NaOH solution at 60℃ in dry oven (Dry-line 10-19870, VWR, EC) for overnight. Subsequently, BC sheet was washed with MQ water repeatedly for several times until the pH was attained, this washing process was going to remove residue NaOH solution from the BC sheet, otherwise it might affect the instruction and composition analysis of BC sheet latter. Then, the BC sheet was incubated in MQ water at 60 ℃ for overnight, this washing procedures were repeated for 3 to 4 times until the alkali was completely washed (pH check with pH paper). After washing, the BC sheet was moved to new pre-weighed petri dish and dried at 60 ℃ in dry oven for

48 hours. The dried BC sheet was weighed by scale. Finally, the BC conversion yield was defined as the ratio of the weight of cellulose obtained to the amount of glucose consumed.

4. RESULTS AND DISCUSSION

4.1 BC production and cells growth of WT and pABCD engineered strain in HS medium and MA/9 minimum medium.

In this experiment, the WT of *K. xylinus* and pABCD was cultivated in two different culture medium- HS medium and MA/9 medium. As shown in Figure 9, (A), the WT and pABCD were cultivated in HS medium.

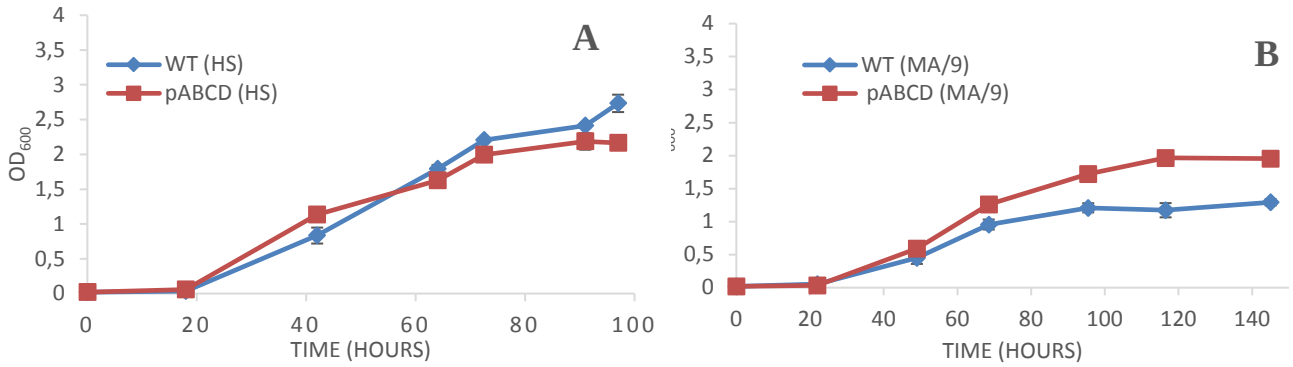


***Figure 9**. Comparison of growth curve between WT and pABCD cultivated in HS medium and MA/9 medium. A) WT and pABCD grew in HS medium; B) WT and pABCD grew in MA/9 minimum medium.*

The results showed that an initial growth rate of pABCD strains was faster than that of WT before 48 hours. After 48 hours, the growth rate of WT increased rapidly and surpassed the pABCD after 64 hours. At the end of cultivation, the growth rate of WT and pABCD decreased gradually to the final value at 97 hours. Even though, the absorbance (OD600) measured for this two type strain did not have significant difference during whole cultivation period in HS medium. It indicates that engineered strain pABCD was able to adapt new culture medium quickly and grow well, compared to WT. In contrast, in MA/9 minimum medium, the result was shown in Figure 9 (B), the growth rate of pABCD increased gradually and much faster than that of WT before 96 hours, after that the growth rate of WT decreased rapidly to the final cell density (OD600) around 1.2 from 96 to 145 hours, but the growth of pABCD start to decrease from 117 hours to 145 hours. In Figure 9, it indicates that WT and pABCD both grow slowly in MA/9 minimum medium containing limited nutrient.

Final OD_{600} of pABCD in HS and MA/9 medium was reached to approximately 2.17 and 1.95, respectively, compared to that of WT were 2.73 and 1.71, respectively. Thus, it indicates that the growth of pABCD did not have significant difference between in HS and MA/9 culture medium, contrasted to that of WT. The possible reason was that 2%

glucose as carbon source supplemented in HS medium was consumed too much to support cells growing at all. Meanwhile, a large amount of gluconic acid accumulated in HS medium resulted in pH dropping down rapidly, the low pH of culture medium could inhibit the cells growth, which has been reported (Kuo et al. 2015).

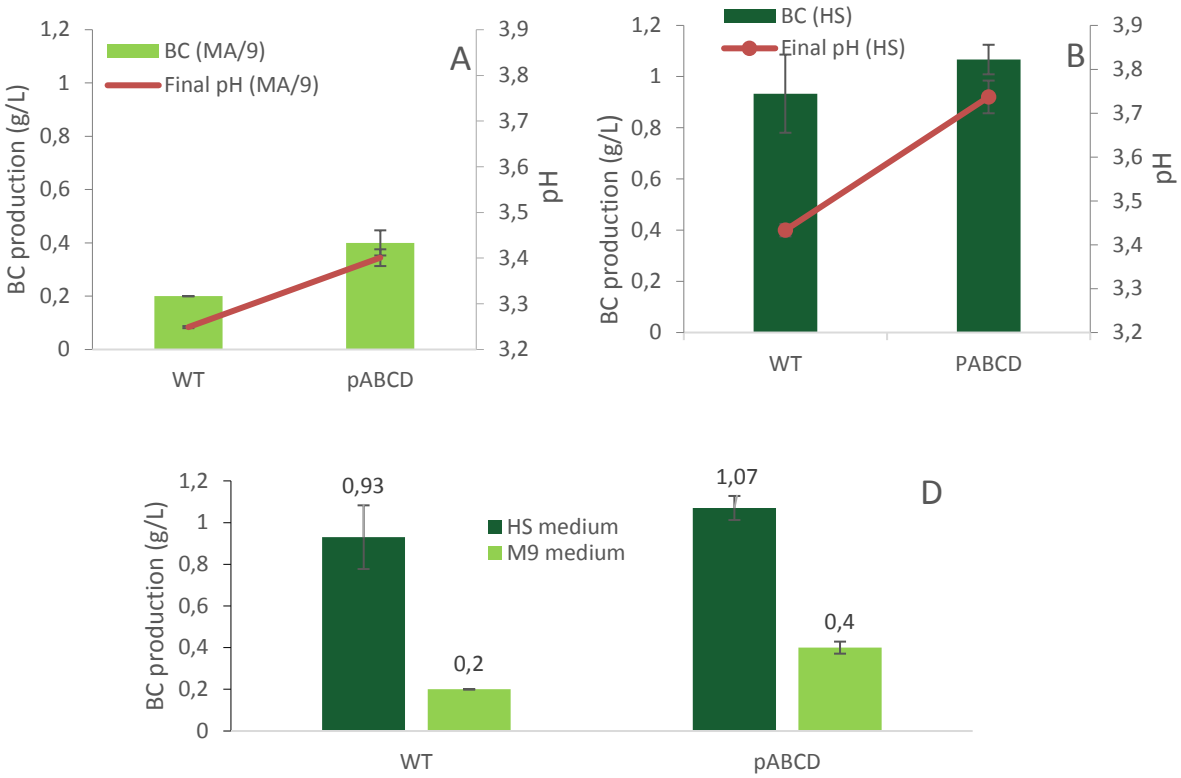


***Figure 10**. Comparison of BC production and final pH between WT and pABCD cultivated in HS and MA/9 medium. (A) Final BC production and pH of two strains in MA/9 minimum medium. (B) Final BC production and pH of two strains in HS medium. (C) Comparison of BC production and final pH between these two strains in two different culture medium.*

There were several steps of BC treatment needing to be employed after end of cultivation. After that the dried BC sheet was obtained and weighed by scale. The amount of BC production was shown in Figure 10, in MA/9 medium, BC production of pABCD was 0.4 g/L after 5 days, almost 2- fold higher than that of WT, and the final pH of culture medium measured for WT reached to 3.2, lower than that of pABCD 3.4 (see Figure 10 (A)). Evidently, the low pH value of culture medium did inhibit the BC production (Kuo et al. 2015).

Similar to previous results, in HS medium, pABCD produced more BC approximately 1.07 g/L after 4 days than that of WT, about 0.93 g/L. The BC production of pABCD did not have significant difference compared with that of WT in HS medium. Moreover, the final pH of WT cultivation and pABCD cultivation reached to approximately 3.4 and 3.7,

respectively (figure 10 (B)). It indicates that the low BC production was accompanied with low pH of culture medium during BC production phase.

According to Figure 10 (C), the WT produced 0.93 g/L in HS medium, almost 5-fold more than that in MA/9 medium 0.2 g/L. The mainly reason was that rich nutrient substance (e.g. yeast extract, peptone etc.) was contained in HS medium, compared to MA/9 minimum medium (absenting yeast extract and peptone substance). Son HJ et al. (2001) pointed out that the yeast extract was the best source resulting in high BC production followed by peptone. Therefore, WT produced obviously more BC production in HS medium than that in MA/9 medium. Nevertheless, the yeast extract and peptone are not the cheaper organic source, so it is economically unfeasible to use them in culture medium for BC production with low cost. In contrast, there was no significant difference of BC production between in HS and MA/9 medium, pABCD produced almost 2.5-fold more BC in HS medium compared with that in MA/9 medium.

The MA/9 medium was evaporated away easier than HS medium when doing BC production at 30℃ in static incubator, due to the lower concentration of nutrient contained in MA/9 medium. The evaporation phenomenon was showed in Figure 11. Since MA/9 medium was evaporated away, it results in changing of medium concentration and pH, and thus it could inhibit the cells growth and decrease the BC production. Therefore, WT and pABCD produced less BC in MA/9 medium than that in HS medium, evaporation of MA/9 medium occurring might be one of probable reasons.

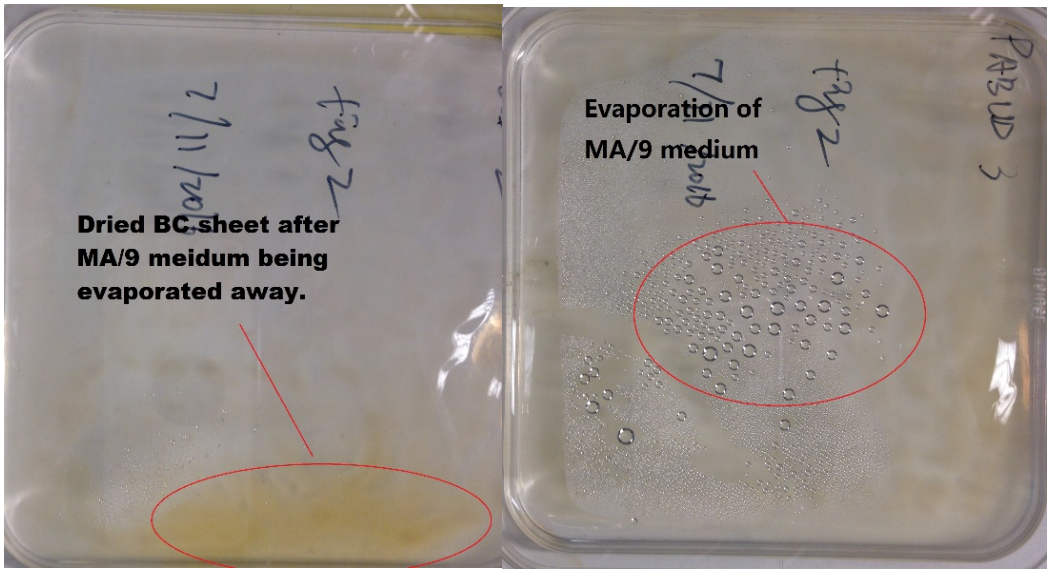


Figure 11. *The evaporation of MA/9 medium occurred in BC production process.*

In addition, the result of HPLC analysis was shown that the gluconic acid accumulated concentration from pABCD and WT was much higher than the initial glucose concentration due to the medium evaporation occurring. Thus, it was hard to say that which one accumulated more gluconic acid at the end of cultivation, it depended on how fraction of medium was evaporated away. But, one important result obtained by HPLC analysis was that 2% glucose supplemented in these two different culture medium was consumed completely by WT and pABCD cells and those glucose was converted into gluconic acid after 4- 5 days cultivation.

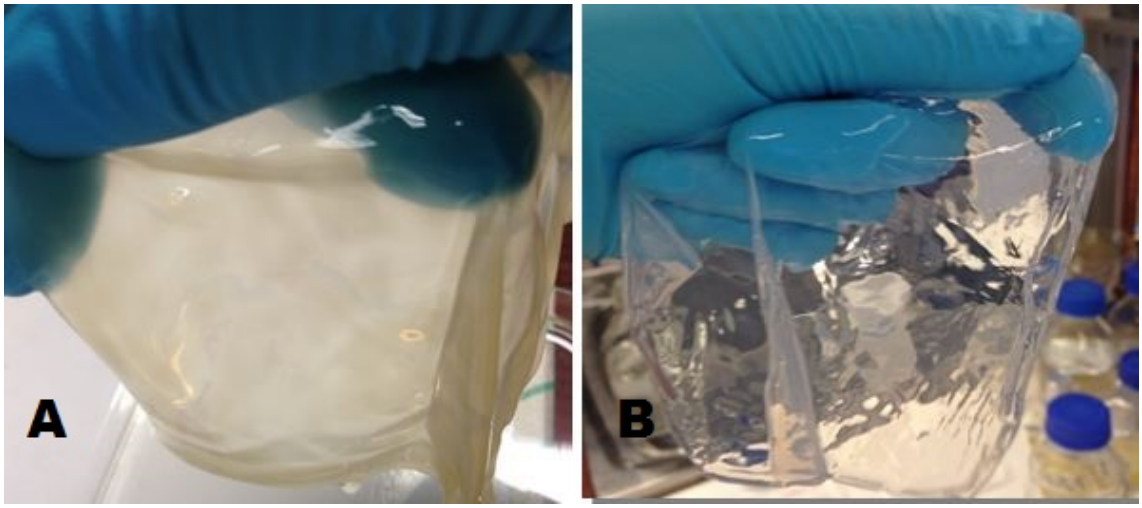


Figure 12*. Wet BC sheets produced by K. xylinus pABCD in HS medium (A) and in MA/9 medium (B).*

As can be observed from Figure 12, without washing treatment of BC sheet, the wet BC sheet produced in HS medium (A) was thicker and deeper brown color than that in MA/9 medium (B). The probable reason was that HS medium contained enough nutrient substance than MA/9 minimum medium, which might be beneficial for BC biosynthesis and cells growth. Hence, a brown layer of pABCD cells was clearly observed on the surface of BC sheet, compared with that of MA/9 medium.

1.1 Optimization of glucose concentration for obtaining high BC production.

As mention above, the glucose is widely used as carbon sources for the cultivation of cellulose-producing bacteria. It plays a significant role in BC production, and the formation of gluconic acid can be problematic (Kuo et al. 2015). When the glucose was used, the gluconic acid will be accumulated as by-product during cultivation of bacteria (Kuo et al. 2015). Hence, pH decrease of culture medium will in turn affect the BC production. Therefore, the initial concentration of glucose supplemented in culture medium is an important factor for BC production (Lee K.Y *et al.* 2014).

Moreover, the cost of glucose is fairly expensive (Keshk and Sameshima 2006) and more gluconic acid accumulated will inhibit the BC production by decreasing the pH of culture medium rapidly. Thus, the optimize glucose concentration for BC production of *K. xylinus* engineered type (pABCD) should be examined in this experiment. As considering the evaporation of incubator effect, we use a new incubating method to avoid this situation.

As we can see from Figure 13, in the almost enclosed plastic box, the MQ water will be evaporated more rapidly than MA/9 medium, and it support enough moisture condition in plastic box. This strategy will benefit and enhance the BC production in 30℃ static incubating condition by avoiding the evaporation away of MA/9 medium. After 5 days incubation, the results was shown that the MA/9 culture medium left in petri dish was much compared to last experiment without plastic box. Therefore, in the next several experiments, this incubating strategy was employed to produce BC in 30℃ static incubator.

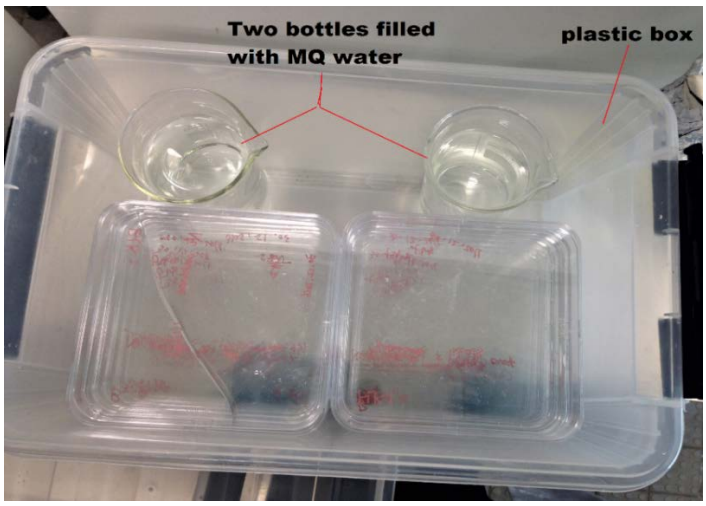


Figure 13*. In 30 ℃ static incubator, all prepared culture petri dishes and two bottle filled with MQ water were placed into one plastic box, and covered a plastic cover on top of box.*

As mentioned earlier, glucose concentration tested in this experiment are shown as follows: 0%, 0.5%, 1.5%, 2%, 3.5% and 5%. In BC production experiment, the pABCD was cultivated in MA/9 medium supplemented with certain concentration of glucose. The different concertation of glucose was employed to investigate its effects on BC production and pABCD cells growth.

As can been seen from Figure 14, after 5 days cultivation, the results showed that the amount of gluconic acid accumulated increased with the increasing amount of initial glucose supplemented in culture medium. All of glucose was consumed completely after 5 days cultivation, and the concentration of gluconic acid accumulated was slightly lower than the initial glucose concentration added in culture medium. The probable reason is

that most amount of initial glucose was used as substance for synthesis of BC and converted into gluconic acid product during the BC production process, and less amount of glucose was used to generate ATP utilized as energy source, which has been reported in some of publications (Lee KY et al 2014).

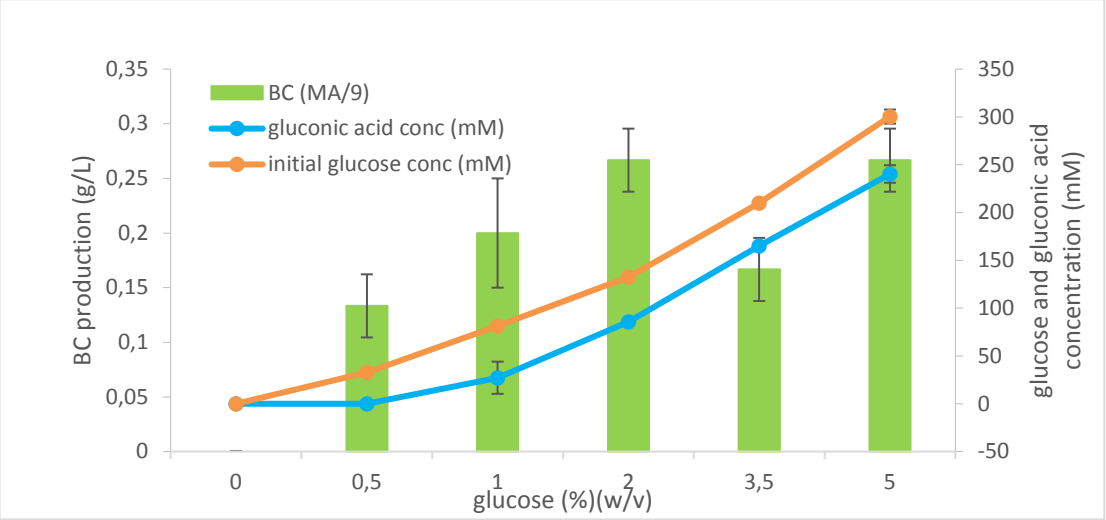


***Figure 14**. The change of BC production, initial glucose concentration supplemented and final gluconic acid concentration accumulated with certain concentration of glucose added in culture medium.*

It is interesting to note that BC production increased with the increasing concentration of glucose between 0-2% range, and the amount of dried BC production were up to 0 g/L, 0.133 g/L, 0.2 g/L, 0.27 g/L, respectively. The gluconic acid accumulation increased simultaneously with increasing of concentration of glucose, and the BC production of these four group increased. However, there was no obvious gluconic acid accumulated when the glucose concentration was 0%. But, the inhibition of gluconic acid effect become more significant as the glucose concentration increased to 3.5% and 5%, the BC production was went down and reached to 0.167 g/L and 0.267 g/L, respectively, which was lower than that of 2% glucose. It might be caused by the high concentration of gluconic acid accumulation. This inhibition effect reduces the BC production by changing the pH of culture medium (Chawla et al. 2009)

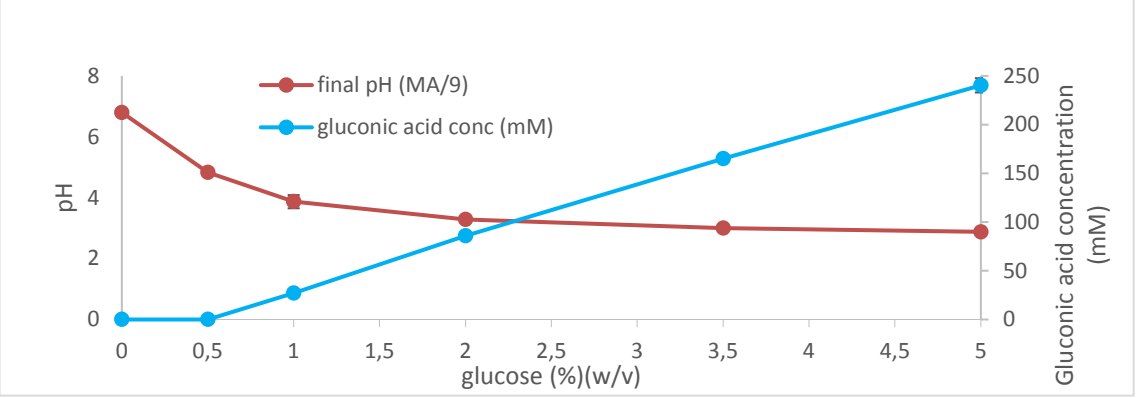


***Figure 15**. After 5 days cultivation, the final pH change curve compared with the concentration curve of accumulated gluconic acid at different concentration glucose in MA/9 culture medium.*

As can be observed from Figure 15, it illustrates that the final pH of culture medium decreased with the increasing concentration of gluconic acid accumulated. At 3.5% and 5% glucose, the final pH of culture medium decreased to 3 and 2.88, respectively. In contrast, the final pH of 0-2% concentration of glucose groups, was 6.8, 4.83, 3.87 and 3.28, respectively, which was higher than that of two other higher glucose concentration group (3.5% and 5%). In addition, the lowest final pH (about 2.88) and highest concentration of gluconic acid accumulated (approximate 240.33 mM) was observed at 5% glucose group.

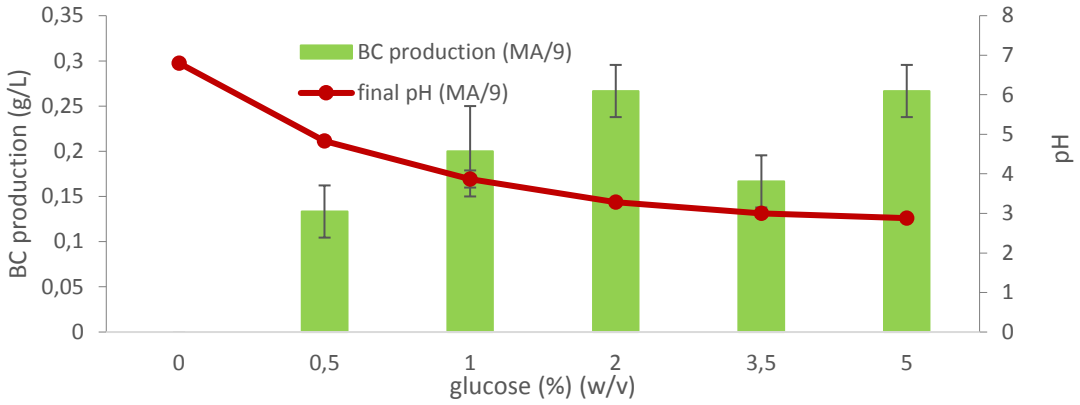


***Figure 16**. The curve of final pH of culture medium compared with the dried weight of BC production.*

The dried weight of BC production was observed in Figure 16. It was found that the final pH of culture medium dropped to below 3.0 in 3.5% and 5% glucose group, the BC yield was more significant inhibited than that of low concentration of glucose (0-2%). Some of publications pointed out that the highest BC production was obtained at pH ranges of between 4.5 and 7.5 (Son HJ et al. 2001). Otherwise, it resulted in lowest BC production when pH of culture medium was below 4.0 (Son HJ et al. 2001). However, low pH can be able to avoid contamination of medium during BC culturing (Son HJ et al. 2001). And the low pH was resulted from the high amount of gluconic acid accumulation during BC biosynthesis process (Kuo et al. 2015). Masaoka et al. (1993) has pointed out why gluconic acid concentration increased with high initial concentration of glucose and resulted in low yield BC. Because the total BC and gluconic acid production equals to the amount of consumed glucose, when the glucose concentration was too high, it was not used to BC biosynthesis, and it would be metabolized gluconic acid to other substances (Masaoka S et al.1993). Therefore, for pABCD strains, higher initial glucose concentration was not able to yield more BC.

All in all, the effect of glucose concentration on BC production by pABCD was investigated in this study, combined all of results showed above, it can be concluded that BC production do be inhibited significantly, and the BC production decreased with increasing initial glucose concentration in culture medium, when the initial concentration of glucose was above 2%. But, the amount of BC production was enhanced and increased when the initial glucose concentration was less than or equal to 2%, and it has been pointed out by Son et al (2003).

It was found that the dried weight of BC production by pABCD strains was about 0.267 g/L when initial concentration of glucose was 2% higher than that of other concentration of glucose group tested. Hence, 2% glucose was considered to be relative optimized concentration of glucose supplemented in culture medium for BC production from *K. xylinus* pABCD strains, and it can be employed in next experiment

1.2 Effects of arabinose and initial density of inoculum on BC production

As can be seen from Figure 17, the results showed that the BC production obtained 0.267 g/L in MA/9 medium added 1% arabinose after 5 days cultivation, 1.6-fold higher than that of culture medium without addition of arabinose (0.167 g/L).

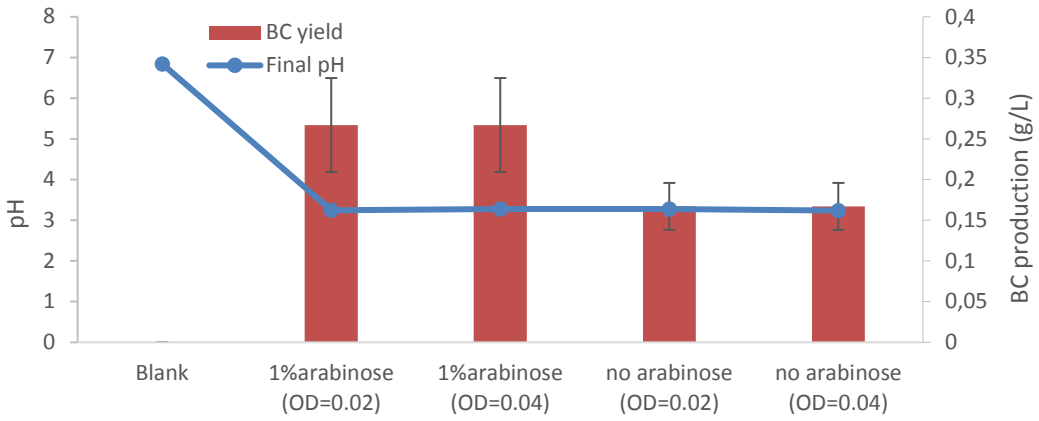


Figure 17*. The effect of the arabinose supplemented in MA/9 culture medium and inoculum volume of pABCD on BC production.*

Hence, the BC production from *K. xylinus* pABCD was enhanced and increased when 1% arabinose present in MA/9 culture medium, compared to that of non-addition of arabinose in culture medium. The probable reason is that pABCD strains contains arabinose promoter gene, thus, the presence of arabinose is able to induce the expression of BC synthetic bcs operon genes, which leads to an increase of BC production (Mangyil et al. In press). In contrast, the absence of arabinose, it means that there is no inducer, it will cause

the expression of BC synthetic bcs operon genes failing, so the engineered strains was supressed into non mutant (as WT) when used for producing BC (Mangyil et al. In press).

The effect of inoculum initial density of strains on BC production was also investigated in this experiment, and the results indicated that the MA/9 culture medium inoculated initial optical density of pABCD at 0.02 and 0.04, produced the same amount of BC production. Therefore, it could consider that initial density of *K. xylinus* pABCD (at 0.02 and 0.04) inoculated in culture medium does not affect the amount of final BC production. Further, it was found that the final pH of culture medium was almost not obviously affected by the addition of arabinose and initials OD of *K. xylinus* pABCD (at 0.02 and 0.04), it was decreased from initial pH (6.8) to approximately 3.2-3.3 range at the end of cultivation.

1.3 Growth of *A. baylyi ADP1Δ gcd* in MA/9 minimum medium supplemented with different concentration of gluconate.

According to experiments above, the maximum concentration of gluconic acid accumulated in BC production process was known. In order to characterize the growth of *A. baylyi ADP1Δ gcd* consumed gluconate in MA/9 medium, we cultivated the *A. baylyi ADP1Δ gcd* cells in MA/9 medium supplemented certain concentration of gluconate as follow (mM=mmol/L): 0 mM, 10 mM, 30 mM, 50 mM, 80 mM, 110 mM and 200 mM. As can be seen from Figure 18, it was found that the *A. baylyi ADP1 Δ gcd* grew slowly during beginning (0-18 h) of the cultivation.

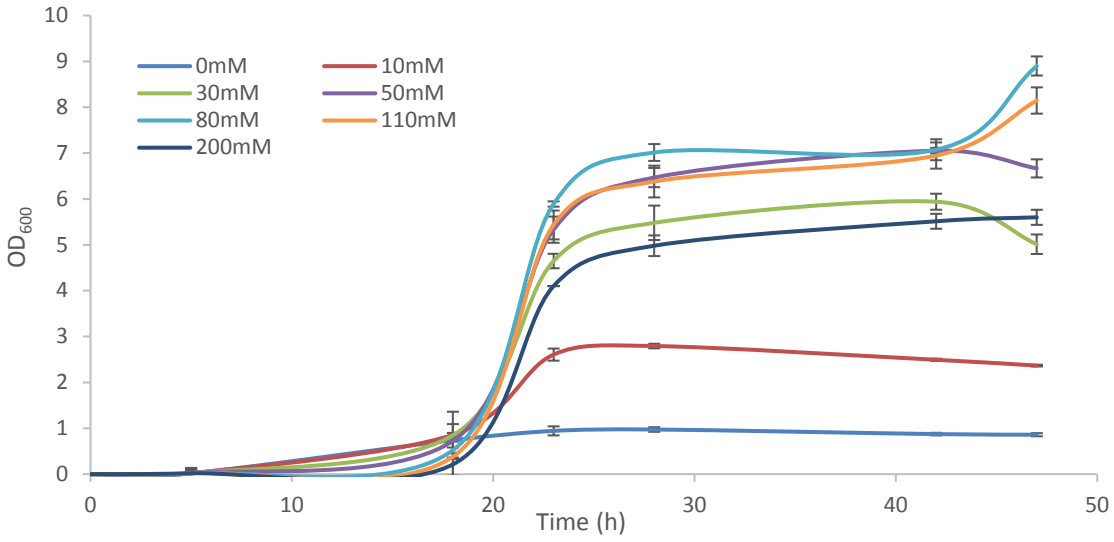


Figure 18. *The A. baylyi ADP1 Δ gcd grow in MA/9 medium supplemented certain concentration of gluconate 2 days.*

The probable reason was that the MA/9 culture medium could induced the *A. baylyi ADP1 Δ gcd* to adapt new cultivation environment, which resulted in low growth rate. But, it

was interesting to note that the growth rate of *A. baylyi ADP1 Δ gcd* went up rapidly in culture medium added 30 mM, 50 mM, 80 mM, 110 mM and 200 mM concentration of gluconate during 18-26 hours cultivation. And we found that the growth rate of *A. baylyi ADP1 Δ gcd* increased with the increasing concentration of addition gluconate (range from 0-80 Mm) in MA/9 medium at this period. Therefore, the growth rate of *A. baylyi ADP1 Δ gcd* was depended on the concentration of sodium D-gluconate in culture medium, which has been reported by Kannisto M et al (2014). The probably reason was that the higher concentration of gluconate was consumed by *A. baylyi ADP1 Δ gcd,* the more cells were generated. But, we also observed that the optical density of cells at 110 mM and 200 mM gluconate was lower than that of 50 mM and 30 mM gluconate, respectively. Because, when the concentration of gluconate was too high to be utilized completely by *A. baylyi ADP1 Δ gcd,* e.g. at 110 mM and 200 mM, it resulted in inhibition of cell growth.

After 42 hours cultivation, the optical density of cells in low concentration gluconate medium (0-50 mM) slightly decreased. In contrast, the optical density of cells in high concentration of gluconate started to increase again. The probable reason was that there were many of cells dead due to the shortage of gluconate in low concentration gluconate culture medium. While, in high concentration gluconate culture medium, it still residue enough amount of gluconate not to be utilized.

As can be seen from *Figure 19*, it was found that all of gluconate was consumed completely by *A. baylyi ADP1 Δ gcd* after 47 hours, when the concentration of addition gluconate in culture medium was between 0 mM to 80 mM range.

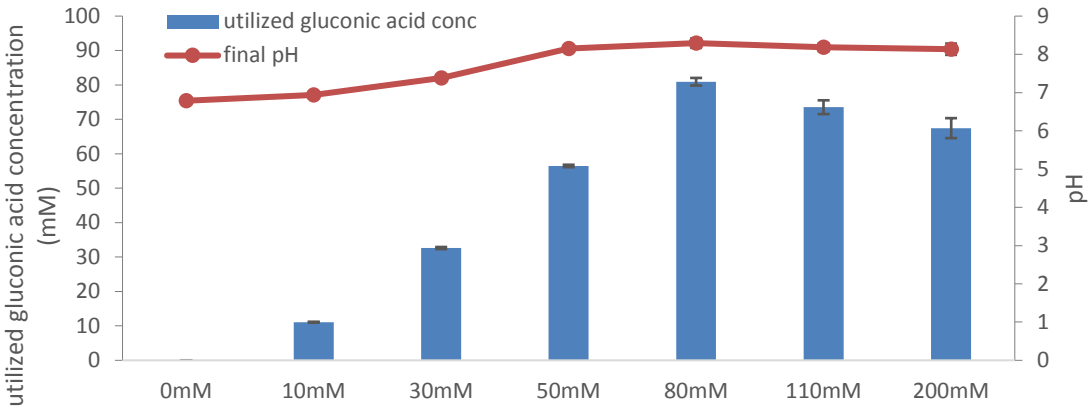


Figure 19. *The final pH of culture medium and gluconate concentration consumed by A. baylyi ADP1 Δ gcd after 2 days cultivation.*

In contrast, there was only 73.5 Mm and 67.4 Mm gluconate consumed by *A. baylyi ADP1 Δ gcd* after 47 hours cultivation in 110mM and 200mM of the initial gluconate concentration culture medium, respectively. This result indicates that *A. baylyi ADP1 Δ gcd* only

consumed approximately 67% and 33.7% in MA/9 culture medium supplemented 110 mM and 200mM gluconate, respectively. Since the inhibition of gluconate consumed by *A. baylyi ADP1 Δ gcd* for cell growth was induced by the excess high concentration gluconate addition in culture medium. Hence, the maximum concentration of gluconate was able to be consumed by *A. baylyi ADP1 Δ gcd* in MA/9 medium was around 80 mM. And the maximum concentration of gluconate utilized might be increased more, if the growth culture time was prolonged. According to the glucose concentration experiment above, 2% glucose was completely consumed and converted into about 85 mM gluconic acid by pABCD strains in MA/9 medium after 5 days cultivation, Therefore, we predicted that gluconic acid accumulated in MA/9 medium added 2% glucose, was able to be consumed completely by *A. baylyi ADP1 Δ gcd*, when the *K. xylinus* pABCD was co-cultivated with *A. baylyi ADP1 Δ gcd* for BC production.

The results shown in *Figure 19* indicate that the final pH of culture medium was similar, being between pH 8.138 and 8.294 from initial pH 6.8, when *A. baylyi ADP1 Δ gcd* was cultivated in MA/9 medium added 50-200 mM gluconate. It was higher than that of 10 mM and 30 mM gluconate group (around 6.9 and 7.4, respectively). Therefore, it indicates that the final pH of MA/9 culture medium increased slightly from initials pH of 6.8 to around 8.3 after 47 h of cultivation. The probable reason was that by- product accumulated in culture medium caused the pH increased between around 8-9 range, when sodium D-gluconate was consumed by *A. baylyi ADP1 Δ gcd* ((Kannisto et al. 2015).

1.4 Growth and BC production of *K. xylinus* Engineered type co-cultivated with *A. baylyi ADP1 Δ gcd* in MA/9 medium

As mentioned above, high concentration of gluconic acid accumulated was resulted from the glucose utilized by *K. xylinus* pABCD during biosynthesis of BC process. Meanwhile, the high amount of gluconic acid accumulated lead to the pH of culture medium drop rapidly. And then the cells growth and BC biosynthesis was inhibited to a certain extent in this case. Therefore, maintaining the pH of culture medium at optimized range for BC production is important and key task.

Therefore, *K. xylinus* pABCD co-cultivated with *A. baylyi ADP1 Δ gcd* in MA/9 medium for BC production was investigated. As can be seen from Figure 20, absorbance (OD_{600}) of co-cultivation increased immediately and rapidly, compared to that of pABCD cultivation alone.

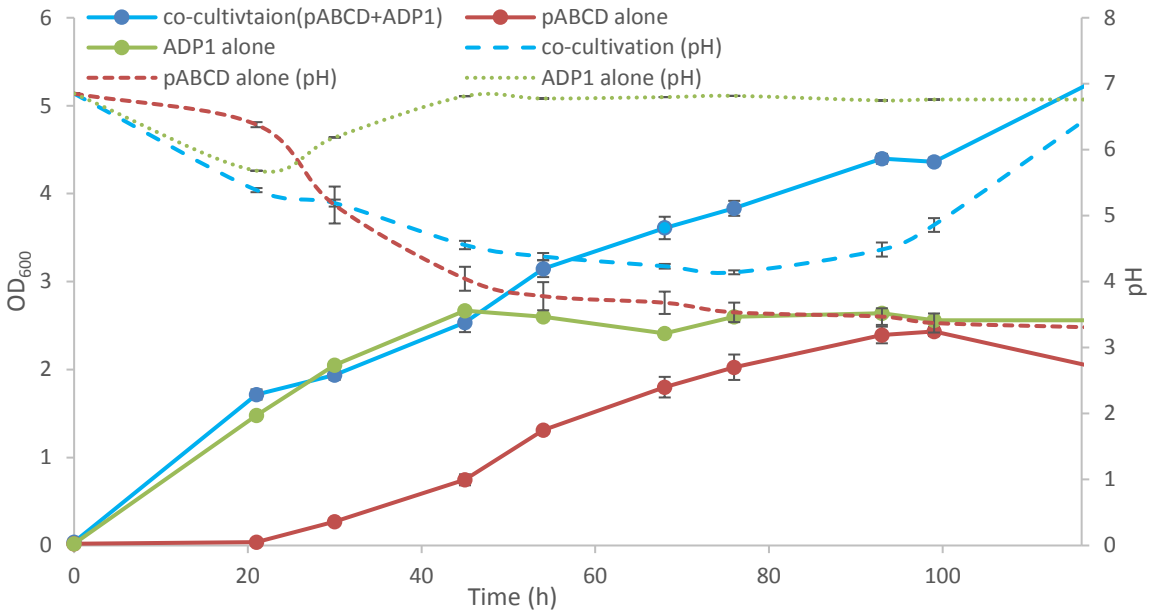


Figure 20. *Cell growth and pH change of co-cultivation and pABCD and A. baylyi ADP1 Δ gcd cultivation alone incubated in MA/9 medium.*

Since there was less amount of LB medium from inoculum left in MA/9 medium, thus, *A. baylyi ADP1 Δ gcd* can utilize it for cells growth. In contrast, at the beginning of 20 h cultivation, the optical density of pABCD almost had not obvious change and closes to 0.02 of initial OD_{600} value. It indicates that the pABCD could prefer to adapt itself to new cultivation environment or stress condition at the beginning of cultivation and grow gradually. And the optical density of *A. baylyi ADP1 Δ gcd* does not obviously increase close to 2.6 after 45 h cultivation due to the absent of carbon source like gluconate in MA/9 medium, in *A. baylyi ADP1 Δ gcd* cultivated in MA/9 medium alone group.

After 20 h to 99 h cultivation, it was found that absorption OD_{600} of co-cultivation increased gradually and reached to 4.36 at 99 h, and it continually rise to maximum value up to 6.76 at 117 h. Meanwhile, the pH of co-cultivation medium was maintained between 4~5 range during this period. After 99 h cultivation, the pH of culture medium started to increase with the increasing of absorption OD_{600} in co-cultivation group. The pH of culture medium could decrease due to the accumulation of gluconic acid, which had been reported by Lee KY et al (2014). Thus, the probable reason was that the accumulation of gluconic acid was consumed by *A. baylyi ADP1 Δ gcd*, which leads to the total absorption OD_{600} increasing and pH maintaining at relative stable level. Further, the stable pH of culture medium was beneficial to the cells growth, compared to that of without co-cultivation group. That is why the total optical density of cells in co-cultivation group increase, when the pH was maintained at 4-6.5 range during cultivation. In other words, the co-cultivation do play an efficient role in keeping pH of culture medium at optimized range 4-7.

In contrast, the results shown in Figure 20 was that the pH of culture medium in pABCD alone group still decreased gradually, which was lower than that of co-cultivation group during the whole cultivation. After 45 h, the low pH of pABCD pure cultivation group was below 4.0. It was resulted from the high amount accumulation of gluconic acid in culture medium, and it was reported that cells growth and BC production can be inhibited at this low pH range in some of extent (Lee KY et al. 2014). Moreover, the pH of culture medium in *A. baylyi ADP1 Δ gcd* cultivated alone group, was not observed significant change during the whole cultivation.

As can be observed from Figure 21, it was interesting to note that there is no any amount of gluconic acid accumulated left in co-cultivation culture medium after 4 days. And the co-cultivation group added 50 μg/ml CAM (diluted with 70% ethanol solution) consumed about 44.5 mM glucose, 6-fold more than that of co-cultivation no CAM addition.

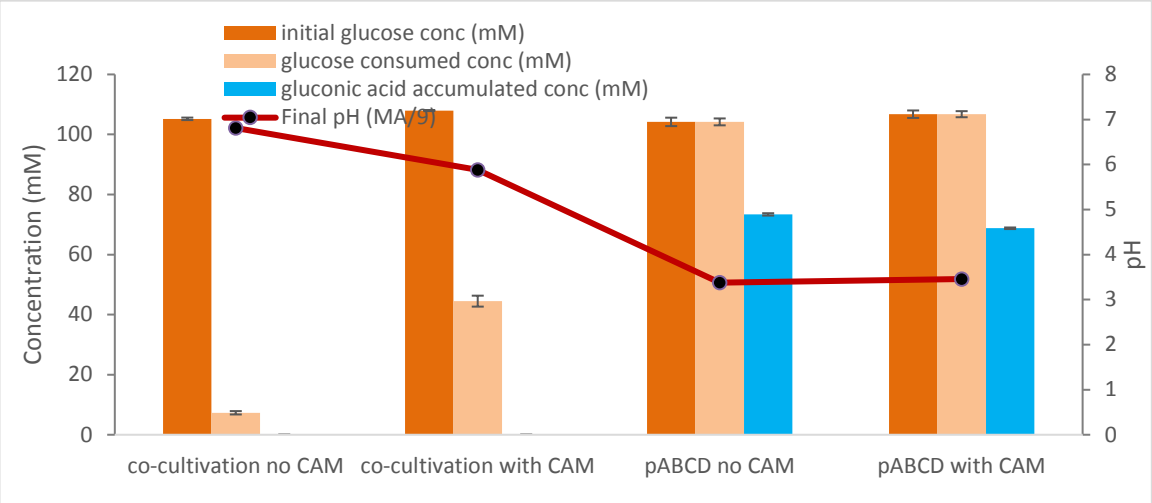


Figure 21. *After 5 days cultivation, the final pH, concentration of glucose consumed and gluconic acid accumulated of co-cultivation group and pABCD cultured alone group in MA/9 culture medium supplemented 2% glucose.*

In contrast, all amount of glucose addition was consumed completely by pABCD cultivated alone in MA/9 medium for BC production after 5 days cultivation. Although there was approximately 73.4 mM and 68.8 mM gluconic acid accumulated by pABCD simultaneously in MA/9 culture medium without and with CAM addition, respectively. And it was interesting to note that the final pH of culture medium decreased with the increasing amount of gluconic acid accumulated, which has been proved by (Kuo et al. 2104).

The result shown in Figure 22 indicates that the dried BC production increased with the increasing of glucose utilized concentration. It was noted that presence of 50 μg/ml CAM was able to enhance the glucose utilization by pABCD for BC production, which resulted in higher BC production than that of CAM absente group.

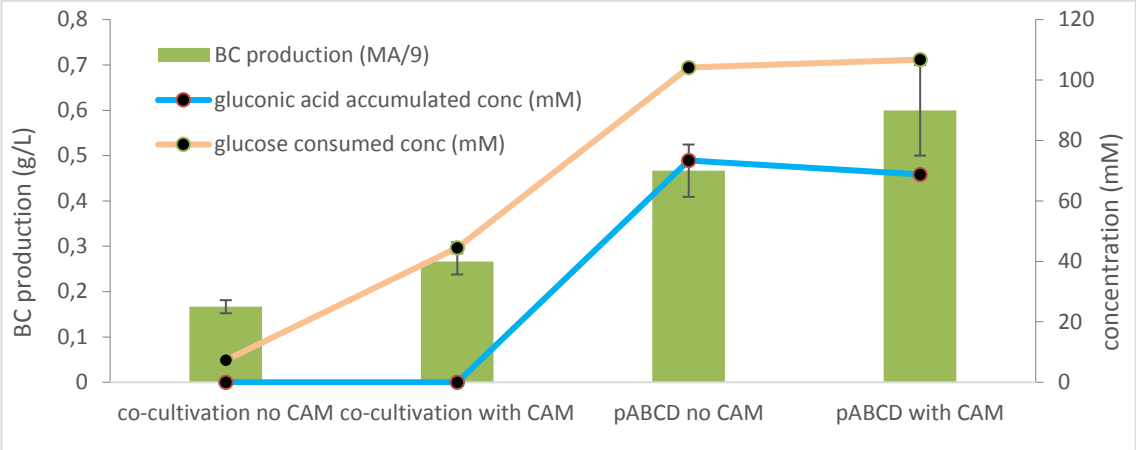


***Figure 22**. Comparison of BC production with concentration of gluconic acid accumulated and glucose consumed in co-cultivation group and pABCD cultivated alone group.*

Once the 50 μg/ml CAM absent, there was only 0.167 g/L and 0.47 g/L BC production in co-cultivation group and pABCD group, respectively. In contrast, when 50 μg/ml CAM presented, the co-cultivation group and pABCD group obtained about 0.27 g/L and 0.6 g/L BC production, correspondingly. It indicates that co-cultivation group obtained less BC production than that of pABCD pure cultivation group, which was not expected result. There are several probable reasons attempted to explain this result. One reason is that *A. baylyi ADP1 Δ gcd* cells could compete with pABCD cells by utilizing micro mineral additive and space in MA/9 medium, when doing co-cultivation experiment for BC production. Other possible reason is that the by-product (see Figure 23 A) & B)) formed from metabolism of gluconic acid by *A. baylyi ADP1 Δ gcd*, might inhibit cells growth of pABCD or BC biosynthesis process. This product was observed from figure 23 A), there was a thick yellow layer on surface of BC sheet after 5 days co-cultivation, and this dark color layer might be some of wax ester by-product produced by *A. baylyi ADP1 Δ gcd*. In contrast, this layer was not appeared in pABCD cultivated alone group.

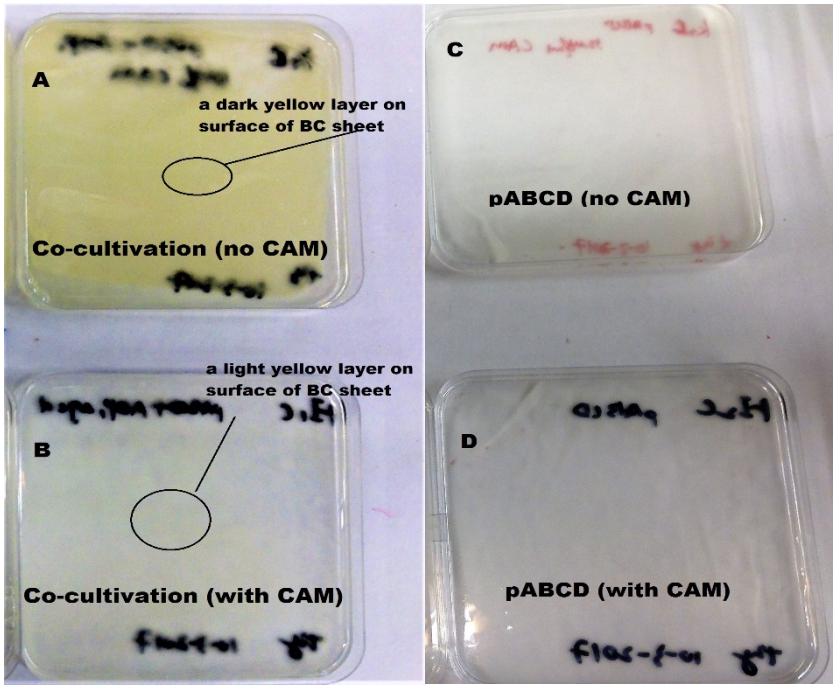


***Figure 23**. A) & B) showed wet BC sheets produced by co-cultivation group without CAM and with 50 µg/ml CAM (CAM), respectively; C) & D) wet BC sheets produced by pABCD group without and with 50 µg/ml CAM.*

As can been observed from Figure 24, the wet and dried BC sheet obtained from pABCD cultivation alone group was thick and has high transparency. Since the BC sheet produced by co-cultivation group was quite less and thin, so, it was damaged easily during washing treatment process and difficult to obtained integrated sheet from co-cultivation group.

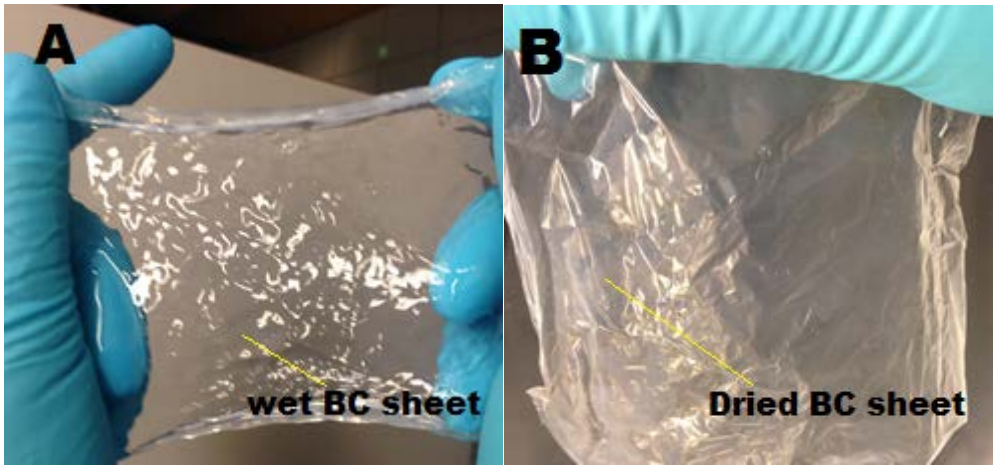


***Figure 24.** The BC sheet produced by K. xylinus pABCD strains from without co-cultivation group. A) Wet BC sheet after washing treatment process; B) Dried BC sheet after washing and drying treatment process.*

As can be observed from Figure 25, in pABCD pure cultivation group, the final pH of culture medium was similar, being between pH 3.377 and 3.454 from the initial pH 6.8.

The mainly reason was that the pABCD consumed all amount of glucose and also accumulated almost same amount of gluconic acid (below 80 mM) in culture medium regardless of presenting CAM or not, after 5 days cultivation.

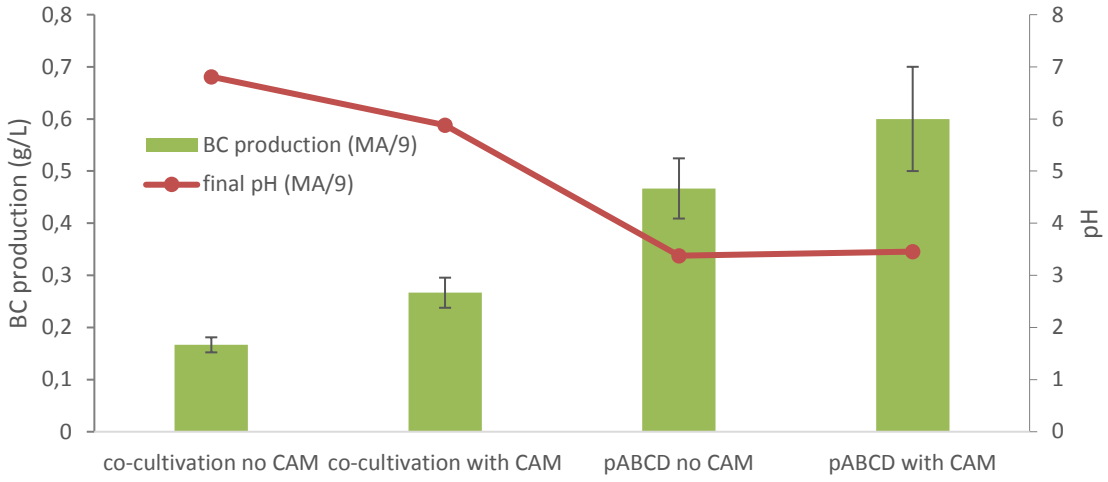


***Figure 25**. Comparison final pH and BC production between co-cultivation and no co-cultivation groups.*

Moreover, in presence of CAM, the BC production increased and reached to 0.6 g/L, approximately 1.3-fold higher than that of without CAM. Since the CAM addition was diluted with 70% ethanol solution, so, the probable reason is that ethanol supplementation contained in 50 μg/ml CAM solution plays a role in the energy supply for ATP generation, which could enhance GHK activity, resulting in improved glucose metabolism for BC production, which has been reported by Shigemtsu T et al. (2005). Further, similar to co-cultivation groups, the BC production on 50 μg/ml CAM in co-cultivation was about 0.267 g/L, around 1.6-fold higher than that of no CAM addition. But, the final pH of culture in co-cultivation without and with CAM almost has no obviously change (around 6.8 and 5.88, respectively), much higher than that of pABCD cultivated alone groups. In co-cultivation groups, the final pH was maintained at the optimization pH range, it should obtain higher amount of BC production than that of pABCD pure cultivation groups. However, it only obtained low BC production, compared to without co-cultivation group. According to this result, we speculate that low BC production of co-cultivation could be resulted from the low glucose utilization in BC biosynthesis process, it was probably caused by the by-product of gluconic acid metabolized by *A. baylyi ADP1 Δ gcd* in co-cultivation culture medium. Certainly, this speculation need to be further discussed and explored in future.

5. CONCLUSION

Bacterial cellulose is increasingly attracting many researchers' and scientists' attention in various industries due to its typical properties. However, the spread commercial applicability of BC is still restricted due to the low BC yield.

A. baylyi ADP1 Δ gcd, was able to grow rapidly by utilizing gluconate directly, but it loss the function of using glucose by removing gcd gene. The aim of this study is to improve the BC production by co-cultivating *A. baylyi ADP1* Δgcd with *K. xylinus* in MA/9 medium. *K. xylinus* has been reported to be able to produce more BC than other bacteria. And the BC production capacity of *K. xylinus* WT and engineered type pABCD was investigated in preliminary experiment. According to these results, it is demonstrated that the WT and pABCD strains was able to grow well and produce BC both in MA/9 medium and HS medium. Even though the BC production observed in MA/9 medium was lower than that in HS medium due to the simple components of MA/9 medium. Since the HS medium was expensive, MA/9 medium was used as economically feasible culture medium for BC production in this study. And it is also demonstrated that pABCD was able to produce more amount of BC than that of WT in MA/9 medium and HS medium. Further, pABCD was also able to utilize glucose as carbon sources for BC production. In this study, it is demonstrated that the optimized concentration of glucose addition in MA/9 medium for BC production was 2%, which resulted in highest BC production compared to that of other different concentration of glucose. Meanwhile, the gluconic acid accumulated around 80 mM, speculated to be consumed completely by *A. baylyi ADP1 Δ gcd,* and it has been proved in gluconic acid consumption experiment.

Moreover, the effect of arabinose on BC production was also demonstrated in this study. The presence of arabinose (1%) was found to be able to induce BC biosynthesis and caused the increase of BC production from pABCD strains in MA/9 medium, and an increase in BC production of nearly 60% was observed, compared to that of arabinose absence. It is also demonstrated that addition of arabinose (1%) does not affect the final pH of static cultivation. Besides that, the initial inoculate volume (OD_{600} =0.02 or 0.04) also does not affect the amount of final BC production.

To further enhance the BC production by eliminating the gluconic acid inhibition to maintain pH of culture medium, the co-cultivation was employed to improve the BC production. This study demonstrates that the pH of cultivation was maintained at optimized pH range (4.0-7.0) by co-cultivation, and accumulation of gluconic acid was speculated to be utilized completely by *A. baylyi ADP1 Δ gcd* at the end of co-cultivation, when glucose (2%) was supplemented in cultivation. However, the low BC production was obtained, which might be resulted from the low glucose utilization, and by-product of *A. baylyi*

ADP1 Δ gcd metabolizing gluconic acid also was considered to be the other possible reason. Certainly, this speculation need to be further discussed and explored in future. Therefore, the next step should focused on how to reduce the negative effect of by-product and increase glucose utilization in co-cultivation, which could be beneficial for enhancing the BC production in some extent. As *A. baylyi ADP1 Δ gcd* was proved to be able to maintain the pH of cultivation by consumed gluconic acid, it can be applied to eliminate gluconic acid inhibition for improvement of BC production or other research area in future.

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